Final Report to Fisheries Research and Development Corporation (FRDC)

Polyunsaturated fatty acid content and nutritional quality of aquaculture feedstocks

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Project 91/59

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1. Executive Summary

Fatty acids and lipids play an important role in animal nutrition. Lipids in general provide a readily utilised energy source and particular constituents are required for specific purposes. For example, sterols are required as membrane constituents and as precursors for steroidal hormones etc. Essential fatty acids (EFA) are those fatty acids which must be obtained or synthesized in sufficient quantity by animals to maintain their growth and survival. Although requirements for specific fatty acids vary, the major EFA for many marine invertebrates and fish are the polyunsaturated fatty acids eicosapentaenoic acid [20:5(n–3) or EPA] and docosahexaenoic acid [22:6(n–3) or DHA]. These are usually supplied from the animal's diet.

Microalgae are used as live feeds for larval crustaceans, for all growth stages of molluscs and as food for prey species such as rotifers and brine shrimp that are used in turn to feed crustaceans and larval fish. The microalgae must supply a balanced proportions of total lipid, protein, carbohydrate and minerals plus essential vitamins, polyunsaturated fatty acids, amino acids, sugars and other micro nutrients. The microalgae are typically grown in large scale batch culture at the hatcheries and provided as needed to the animals. This is expensive and requires dedicated facilities and specialist expertise. Almost all hatcheries now use starter cultures provided by the CSIRO algal culture facility operated by the Division of Fisheries.

This report reviews the fatty acid and lipid compositions of the major classes of microalgae used by the mariculture industry in Australia and elsewhere. These data were obtained as part of the FRDC–funded study "Polyunsaturated fatty acid content and nutritional quality of aquaculture feedstocks" (grant 91/59) and complement data obtained from its predecessor FIRTA–funded study "Essential fatty acid content of feedstocks used in aquaculture" (grant 88/69).

Our research has shown that there is considerable variability in the lipid composition of microalgae. Some species are rich in triacylglycerols while in others almost all of the fatty acids occur as polar lipids such as phospholipids. The protein content of most microalgae is high and the amino acid composition is remarkably uniform. However, carbohydrate content is highly variable and there are significant differences in sugar composition between species. The relative proportions of major fatty acids also vary greatly from one class to another. For example, in diatoms the major fatty acids are 16:0 [1], 16:1 and 20:5(n–3) whereas in chlorophytes C_{18} polyunsaturated fatty acids (PUFA) predominate.

Most diatoms, eustigmatophytes, prymnesiophytes and cryptomonads contain significant amounts of the essential fatty acids eicosapentaenoic acid [20:5(n–3)] and/or docosahexaenoic acid [22:6(n–3)]. Species from these groups often give good results in animal growth trials, although exceptions are known where size, morphology or other

[1]Fatty acids are denoted by x:y(n–z) where x is the number of carbon atoms, y is the number of double bonds and z denotes the position of the double bond closest to the methyl end of the molecule. All double bonds are assumed to be separated by two CH_2 groups unless otherwise stated.

nutritional factors limit their use. Green microalgae from the class Prasinophyceae (prasinophytes) commonly contain smaller amounts of these acids, but have been used successfully as mariculture feeds in some applications. In contrast, most green algae from the class Chlorophyceae lack appreciable amounts of polyunsaturated fatty acids which limits their usefulness as a single species diet. New isolates of *Pavlova* and *Nannochloropsis* species were studied and found to contain high concentrations of PUFA. These species may prove to be good alternatives to currently used species, particularly in tropical hatcheries.

Sterol content and composition is also highly variable although a few classes display characteristic distributions of a limited number of sterols which can be useful for taxonomic assignments. Most contain C_{28} and C_{29} sterols but a few, such as species of *Nannochloropsis*, contain a predominance of the "animal" sterol cholesterol which enhances their suitability as feeds for those animals with limited ability to synthesize sterols.

Several species in common use in mariculture were found to contain significant amounts of unusual lipids. For example, species of *Pavlova* were found to contain steroidal diols (now termed pavlovols), *Isochrysis* contains long–chain unsaturated ketones (alkenones) and eustigmatophytes contain alkyl diols and long–chain alcohols. The nutritional significance of these compounds is unknown, although it seems that they are not deleterious. Note , however, that the alkenones are not assimilated or utilised by many marine animals.

The biochemical composition of microalgae grown in batch culture as practised by most hatcheries is significantly affected by growth conditions. Levels of total protein, carbohydrate and lipids can change at different growth stages or with changes to environmental conditions such as nutrient supply, light intensity or temperature. We have found that for *Nannochloropsis oculata* and *Isochrysis* (T.iso) grown in 100 litre bag culture the amounts of triacylglycerols and total fatty acids were very much higher, and the content of 20:5(n–3) lower, in samples cropped during stationary phase than in the same algae cropped during exponential growth. Such observations show that the algal growth conditions can be manipulated to optimise the content of energy–providing fats and other biochemicals.

A study was conducted of the fatty acids in the oyster *Crassostrea gigas*. Adult oysters from 2 different size classes showed little difference in the amounts or composition of lipid classes, fatty acids or sterols. Major fatty acids were 16:0, 20:5(n–3) and 22:6(n–3); the abundance of the latter PUFA was particularly high and represented over 35% of the total fatty acids. Small amounts of non–methylene interrupted PUFA were also detected. During this work a problem was encountered with poor extraction of lipids from lyophilized samples (i.e. those that had been freeze dried) using conventional methods. We discovered that the samples had to be rehydrated before extraction to ensure good recovery of lipids. This is a common preservation technique for animal tissues and it is likely that some lipid and fatty acid results in the literature are biased because of this problem.

The lipids and fatty acids of microalgae can be transferred to zooplankton which in turn can be used as feedstocks for juvenile animals. The contents of 20:5(n–3) and 22:6(n–3)

in rotifers *Brachionus* can be boosted by feeding them on microalgae such as *Pavlova* or on artificial feeds such as Superselco, but levels of 22:6(n–3) in the brine shrimp *Artemia* remain low even when fed microalgae rich in this fatty acid. This can be a problem for industry since many of the animals being reared require 22:6(n–3) in the diet. In recent years, there has been an increasing interest in finding alternatives to *Artemia* such as copepods and smaller rotifers.

Our research has generated a large number of scientific papers and reports to industry. We have communicated our results widely through personal contacts, articles in the newsletter "Microalgae for Mariculture", and presentations at workshops, training courses and conferences. The importance of PUFA as dietary components is now well recognized by the Australian mariculture industry and a solid base of knowledge has now been made available to assist industry to match the nutritional needs of farmed animals with available feeds and to formulate better feeds for the industry.

2. Background and Research Need

Research funded by this grant commenced on January 1, 1992 and laboratory work was completed on December 31, 1994. This project extended work previously funded by FIRTA grant 88/69 entitled "Essential fatty acid content of feedstocks used in aquaculture" and it was designed to complement research undertaken by Dr S. W. Jeffrey and co-workers on FRDC grant 90/63 entitled "Microalgae for Mariculture". Information on the PUFA content of existing live and artificial feeds used for larval rearing has largely come from overseas studies and hence there was a clear need when the project started to obtain data relevant to Australian conditions. Information on the requirements of different animals for specific fatty acids and other organic nutrients is also needed so that feed quality can be optimised, costs reduced and growth rates increased.

The success of most mariculture operations is critically dependant on achieving good growth and survival of the larval animal at the hatchery stage. One of the major factors is the nutritional quality of the food used to rear the animal. It is well documented that many marine animals require specific dietary polyunsaturated fatty acids (PUFA) including the highly unsaturated acids 20:5(n-3) (EPA) and 22:6(n-3) (DHA) (see Figure 1 for structures) for good growth and survival, particularly during larval and juvenile stages when the animals lack sufficient capacity to synthesize enough PUFA for their needs (Enright *et al.*, 1986a,b; Langdon and Waldock, 1981). Structures of the main PUFA and the biosynthetic pathways by which they are formed are shown in Figure 1. The PUFA source may be microalgae, prey species (copepods, rotifers, brine-shrimps), or artificial foods (powders, pellets, microcapsules). The choice will depend on the particular animal being reared and on its stage of growth. For many of the new species being raised by the Australian industry, appropriate information on nutritional requirements is simply not available and must be obtained by experimentation and feeding trials.

Microalgae provide a suitable live food for most stages of mollusc growth and are used to feed zooplankton that are used in turn as food for larval fish. Adequate nutrition at the larval and juvenile stages is of critical importance to the overall success of the mariculture operation, and to date live foods are still preferred to dry or artificial feeds. Inadequate diets can also adversely affect maternal fecundity and reduce egg viability (e.g. Luquet and Watanabe, 1986). Rodgers and Barlow (1987) showed that larvae of the highly valued fish barramundi (*Lates calcarifer*) suffered high mortality and numerous ailments in culture when feedstocks lacked PUFA. Larvae commonly have low energy reserves and so are easily starved unless given readily digested food of appropriate nutritional value (e.g. Fraser *et al.*, 1988).

Penaeid prawns and shrimps appear to have a limited capacity to synthesize essential fatty acids and so must obtain them from the diet if high weight gains are to be achieved (Kanazawa *et al.*, 1979; Kanazawa, 1985). Marine fish are believed to have an absolute requirement for (n-3) PUFA and possibly (n-6) fatty acids as well (Watanabe, 1982; Tocher and Sargent, 1984; Bell *et al.*, 1986). Fish production can be increased by feeding them on

zooplankton whose nutritional value has been upgraded by being raised on diets enriched in (n–3) polyunsaturated fatty acids (Watanabe *et al.*, 1982; Millamena *et al.*, 1988; Nichols *et al.*, 1989). For example, Witt *et al.* (1984) considered that the high content of 22:6(n–3) in copepods was the main factor contributing to their high dietary value to turbot larvae. Alteration in lipid metabolism and enzyme activities leading to liver damage was noted by Lemaire *et al.* (1991) in sea–bass which had been fed on diets deficient in long–chain PUFA. Owen *et al.* (1975) showed that turbot could not convert 18:2(n–6) fatty acid to 20:4(n–6) when fed corn oil, and that growth was inhibited on a diet containing 18:3(n–3). However, the fish grew well when 20:5(n–3) and 22:6(n–3) were included in the diet.

Arachidonic acid [20:4(n–6); Figure 1] is a significant component of the lipids of certain benthic macroalgae and animals which feed directly on them. These include commercially valuable species e.g. abalone (*Haliotis* spp.) and spiny lobsters (*Panulirus* spp.). Whether 20:4(n–6) is essential to these marine animals needs to be examined for artificial feed formulation. Arachidonic acid may also have a special role in the embryonic and larval stages of fish since it is present in phosphatidyl inositol even at the earliest stages of larval development (Tocher and Sargent, 1984). In most marine animals 20:5(n–3) and 20:4(n–6) are obtained directly from the diet and small amounts of both C_{20} PUFA are converted into the anti–inflammatory leucotrienes and the prostaglandins necessary for growth and reproduction.

There is limited knowledge of sterol metabolism in marine animals, and it appears that most studies have concentrated on mariculture animals, such as crustaceans, molluscs and fish. The sterol composition of fish consists almost entirely of cholest–5–en–3β–ol (cholesterol; Figure 2), which most fish can synthesize *de novo* (Teshima 1991 and references cited therein), and so it is unlikely that they require a source of sterols in their diet. The major sterol found in crustaceans is also cholesterol. These animals do not have the ability to synthesize sterols *de novo*, but they have the ability to dealkylate and convert some C_{28} and C_{29} sterols from their diet to cholesterol to meet sterol requirements (Goad 1978, Teshima 1982, 1991 and references cited therein). Cholesterol is also the major component in the sterol profile of gastropods which have the ability to both synthesize cholesterol *de novo* and to dealkylate dietary phytosterols (Goad 1978, Teshima 1982, 1991 and references cited therein). Bivalves contain complex mixtures of C_{26}, C_{27}, C_{28}, C_{29} sterols as well as cholesterol thought to be due to the complexity of dietary sterols and their slow rate of sterol metabolism (Teshima 1982, 1991 and references cited therein). There is contradictory evidence about the ability of bivalves to synthesize sterols *de novo* and to dealkylate dietary phytosterols, but it appears that they may have only a limited capacity for sterol synthesis (Goad 1978; Teshima 1982, 1991 and references cited therein).

Our work involved a systematic study of the fatty acid and lipid biochemistry of marine microalgae used as mariculture feedstocks as well as related species which might be better suited for use in Australian conditions. The effects of culture conditions on fatty acid composition were also studied so that hatcheries could harvest microalgae of optimum nutritional value. It should be noted that the costs of culturing microalgae may represent 30–50% of operating costs in hatcheries, so any reduction of these costs will be of significant

benefit to the industry. We also aimed to identify feeds with sufficient amounts of the essential PUFA required by the various groups of marine animals (crustaceans, fish and molluscs) so that mariculturists could make informed decisions on which microalgal species and which intermediate zooplankton species (e.g. rotifers, copepods, brine shrimp) are best suited as feedstocks.

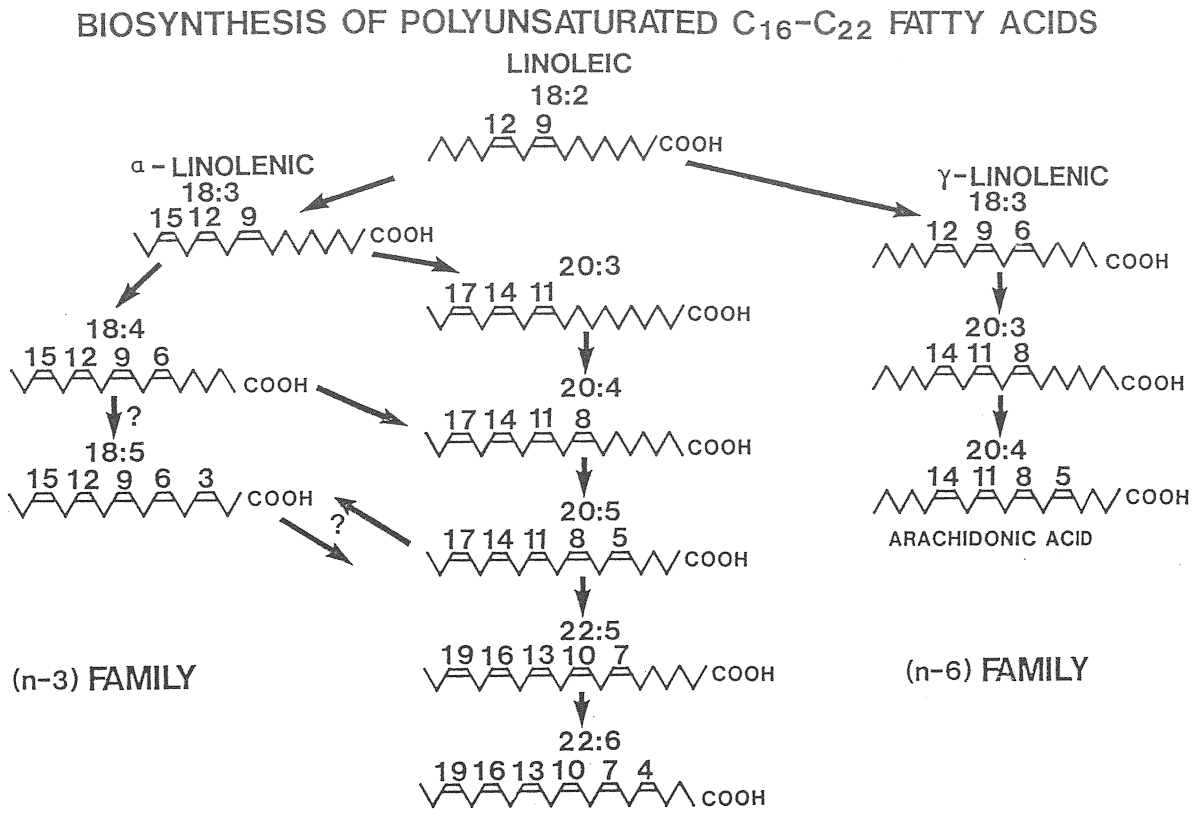


Figure 1 Biosynthesis and structures of the main long-chain polyunsaturated fatty acids

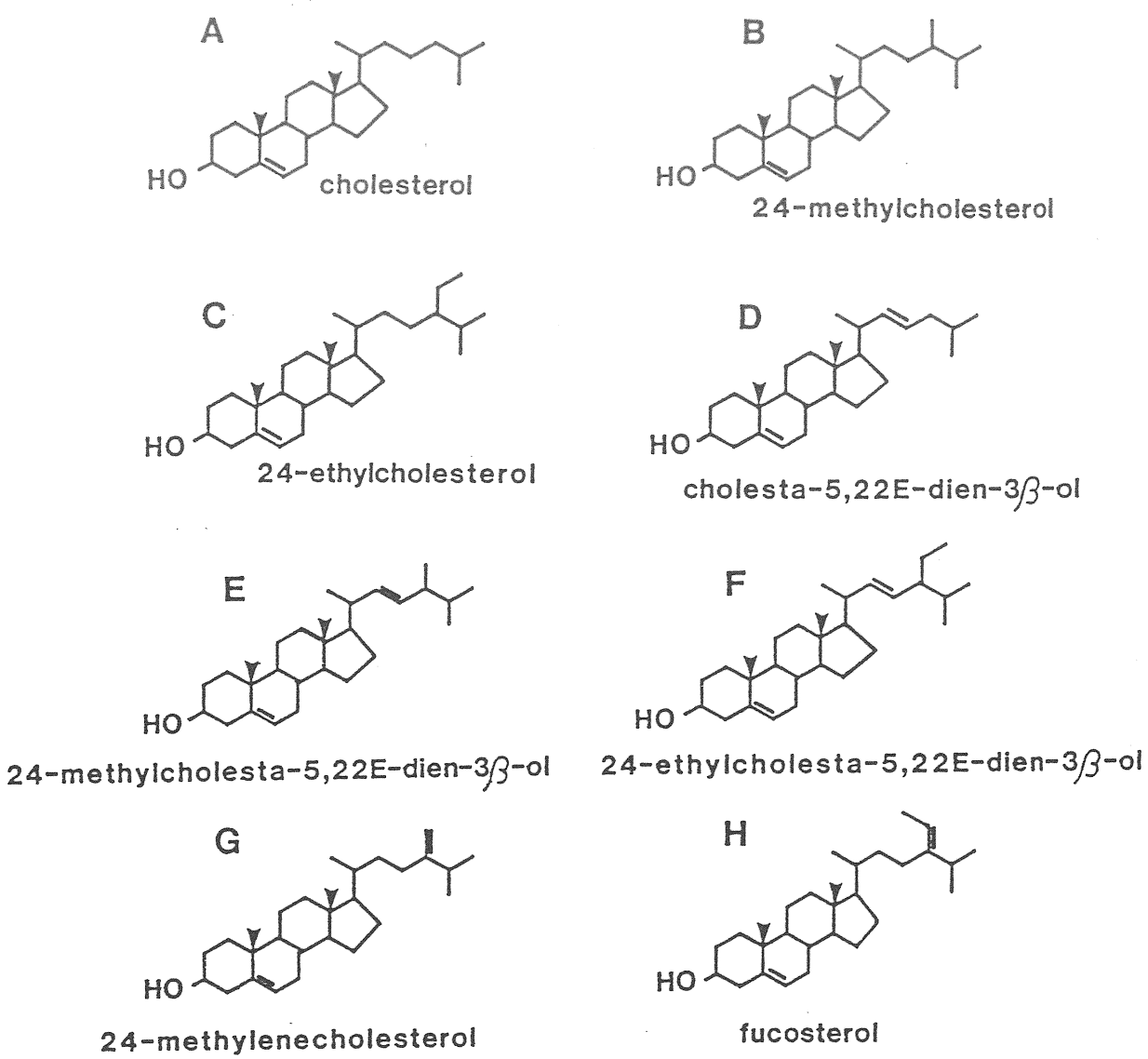


Figure 2. Structures of the 8 most common sterols found in microalgae. Note that cholesterol is the most common sterol in animals.

3. Objectives

The objectives of this project were:

(1) Measure the concentration of essential fatty acids 20:5(n−3) and 22:6(n−3) in microalgae and other feedstocks used in Australian hatcheries.

(2) Survey the lipid and fatty acid content of microalgae to determine characteristic distributions for the major algal classes and to identify new species of use to the Australian mariculture industry, particularly in tropical hatcheries.

(3) Identify the effects of different environmental conditions on fatty acid and lipid composition of microalgae by comparing cultures grown under a variety of defined conditions, and hence determine how to optimize fatty acid content.

(4) Assess the effects of algal feeds with different fatty acid contents on animal production in support of animal growth trials with bivalve molluscs, particularly the oyster *Crassostrea gigas* (to be carried out with FRDC grant 94/83).

(5) Identify the effects of various diets (rotifers and copepods) which have different fatty acid compositions on the growth and survival of fish larvae (with staff from the Tasmanian Division of Sea Fisheries), and crustacean larvae (with University of Tasmania).

(6) Assist the fishing and mariculture industries with advice on nutrition of marine organisms, and provide a service for the analysis of polyunsaturated fatty acids and other lipids in marine samples.

4. MATERIALS AND METHODS

4.1 Phytoplankton Cultures

All algal cultures (Table 1) were obtained from the CSIRO Culture Collection of Microalgae. Most cultures were grown in medium f/2, although others were grown in medium G or specialised media as required. Details of the chemical composition of these media are provided in our publications or cited references (see Jeffrey, 1980). Media were prepared from autoclaved sea water with filter–sterilised nutrients added aseptically; media compositions are detailed elsewhere. All equipment for the cultures (glassware and tubing) was sterilised by autoclaving and all inoculations were performed under aseptic conditions to avoid contamination. Cultures of the tropical species, (e.g. *Fragilaria pinnata* and *Skeletonema* sp.) were grown at 25° or at 28° (e.g. *Thalassionema nitzschioides*); the remaining temperate species were grown at 20° (± 0.5°). Cultures were illuminated from beneath using white fluorescent light (Daylight tubes) on 12:12 hr light:dark cycles. A common light intensity was 70–100 μE m^{-2} sec^{-1}. In the case of some diatoms (*Rhizosolenia setigera*, *Amphiprora hyalina*, and *Thalassionema nitzschioides)* the light was filtered through Belgian signal glass ("blue light", 40 μE m^{-2} sec^{-1}) after 3 days to improve cell densities or cell morphology. Cultures were grown in 700 ml of media in 1 l Erlenmeyer flasks or 1400 ml in 2 litre flasks and were aerated with filtered air (0.2 μm membrane filters) supplemented to 0.5% (v) with CO_2. Aeration was not used in some early work nor for the diatoms *Thalassionema nitzschioides* and *Thalassiothrix heteromorpha* which grew significantly better without aeration. All cultures were harvested towards the end of logarithmic phase.

4.2 Lipid extraction and fractionation

Cells were harvested by filtering culture medium through a 47 mm diameter glass fibre filter (Whatman GFF, nominal pore size 0.7 μm). The filter was briefly air–dried and then extracted 5 times with chloroform–methanol–water (1:2:0.8, 5 ml) using ultrasonication in a modification of the traditional Bligh and Dyer extraction method used for fish lipids. (Bligh and Dyer, 1959) to produce the total lipid extract (TLE). Full details are given in Volkman (1989) and Volkman *et al.* (1989). Chloroform and purified water (Milli–Q® system) were then added to the combined extracts to bring the chloroform–methanol–water ratio to 1:1:0.9. The solvent layers were then allowed to separate. Lipids were recovered in the lower chloroform phase and the aqueous phase containing salts and other water soluble material was discarded. The solvents were removed under vacuum, and the lipid extract was stored under nitrogen at –20 °C until analysed within a few days.

This procedure generally gives excellent recoveries of lipids from most microalgae except for those with tough cell walls. Prolonged extraction with chloroform–methanol did not improve yields, but a pre–extraction with dimethyl sulfoxide followed by 3 extractions with chloroform:methanol:water was usually sufficient to liberate all commonly–

encountered lipids in such cases. The efficiency of the extraction was improved by the use of ultrasonic energy to disrupt the cells. If green coloration was still obvious in the cells after extraction then it was considered likely that some lipids had not been extracted. In such cases, the pellet was saponified to release any remaining fatty acids.

A similar method was used to extract the lipids of marine animals, although some difficulties were experienced with the extraction of lipids from freeze dried samples and some dried artificial feeds; this was particularly severe with lyophilised oyster tissue. After some experimentation it was discovered that the samples had to be rehydrated before extraction. Full details are provided later in this report in section 5.3.

4.3 Analysis of lipid classes

The identities and concentrations of the major lipid classes were determined by analysing a portion of the total lipid extract with an Iatroscan Mk III TH-10 TLC-FID analyser (Iatron Laboratories, Japan), as described by Volkman *et al.* (1986) and Volkman and Nichols (1991). The solvent system used for the lipid separation was hexane-diethyl ether-acetic acid (60:17:0.2) which resolves hydrocarbons, triacylglycerols, free fatty acids and sterols and polar lipids. Individual polar lipids were not identified. This technique permits sub-microgram amounts of lipid classes to be determined without the need for separating and weighing individual lipid fractions. Multiple solvent developments can be employed if desired to separate neutral lipids and individual polar lipids (Parrish, 1987), although this was not used in the work presented here. Typical chromatograms are shown in Figure 3.

4.4 Preparation of samples for detailed analysis

Total fatty acid methyl esters (FAMEs) were obtained by direct transesterification of an aliquot of the total lipids with methanol-chloroform-HCl (10:1:1) or 14% BF_3/methanol. Both methods give similar results (Volkman *et al.*, 1991), but the former method was preferred for routine work. In our experience, yields from direct transesterification can be up to 10% higher than those obtained by the two-step process of alkaline saponification followed by esterification. One disadvantage of direct transesterification is that neutral lipids such as hydrocarbons and sterols are included with the FAME which could cause problems with peak identification if mass spectral data are not available for compound confirmation. Small amounts of artefacts from phytol degradation are also commonly present when solvent systems containing acids such as HCl or BF_3 are used.

For transesterification, an aliquot of the total solvent extract was reduced to dryness under N_2 and reacted with methanol-hydrochloric acid-chloroform (10:1:1; 3 ml) for 2 hours at 80 ^{o}C to esterify free fatty acids and transesterify triacylglycerols and more polar esters. This procedure also liberates any extractable neutral lipids bound by ester, glycosidic or amide bonds to more complex lipid structures. Distilled water (3 ml) was then

added and the reaction products were extracted 3 times with chloroform–hexane (1:4; 3 ml). The alcohol components in the transesterified extract were converted to trimethylsilyl ethers (TMSi–ethers) with bis(trimethylsilyl)trifluoroacetamide (BSTFA) for GC and GC–MS analysis.

To isolate neutral lipids such as sterols, a separate aliquot of the total extract was saponified in 5% KOH in methanol–water (4:1) at 80 ^{o}C for 2 hours. Neutral constituents such as sterols, diols, and alcohols were extracted with hexane:chloroform (4:1) and derivatized with bis(trimethylsilyl)–trifluoroacetamide (BSTFA) to form trimethylsilyl ethers for GC and GC–MS analysis. Total hydrocarbons were isolated from a few samples (e.g. diatoms) by open column chromatography of a portion of the total extract on silica gel and eluted with hexane and hexane:toluene.

Some lipid samples were further separated by thin layer chromatography. For example, a portion of the total extract was separated into neutral lipid classes by thin–layer chromatography on 0.25 mm silica gel G–25 UV_{254} pre–coated plates (Machery–Nagel). The extracts were applied in chloroform and the plate was then developed in hexane:diethyl ether:acetic acid (50:50:1). The plates had been pre–developed in this solvent system to remove any contamination. R_f values were established using appropriate standards. The following representative R_f values were obtained: hydrocarbons (0.71); methyl ketones (0.51); long–chain alcohols (0.43); C_{30}–C_{32} alcohols (0.41); hexadecanol (0.36); 4–methyl sterols and triacontan–15–one–1–ol (0.34); 4–desmethyl sterols (0.27); C_{30}–C_{32} diols – (0.22). Bands were visualised by spraying with 0.1% 2',7'–dichlorofluorescein in methanol and then extracted with chloroform, derivatized with BSTFA and analysed by GC and GC–MS.

Many of the commonly encountered positional isomers of monounsaturated FAME can be separated by capillary chromatography, but minor isomers are best identified using double bond derivatization techniques such as the formation of dimethyldisulfide (DMDS) adducts (Nichols *et al.*, 1986). Unsaturated fatty acids were also separated according to their degree of unsaturation by argentation TLC when required and identified using DMOX derivatives (Dunstan *et al.*, 1993a and refs therein).

Carbon number chain–lengths and the presence of branching in fatty acids, sterols or fatty acids were confirmed by hydrogenation. Adams catalyst (PtO_2) was added to a flask containing iso–octane (2 ml) which was then flushed with H_2 for 5 min. An aliquot of the sample in isooctane was then added and H_2 was bubbled through the suspension for 3 hours. After addition of distilled water (2 ml), the hydrogenation products were removed in the upper isooctane layer (4 extractions) and analysed by GC and GC–MSD.

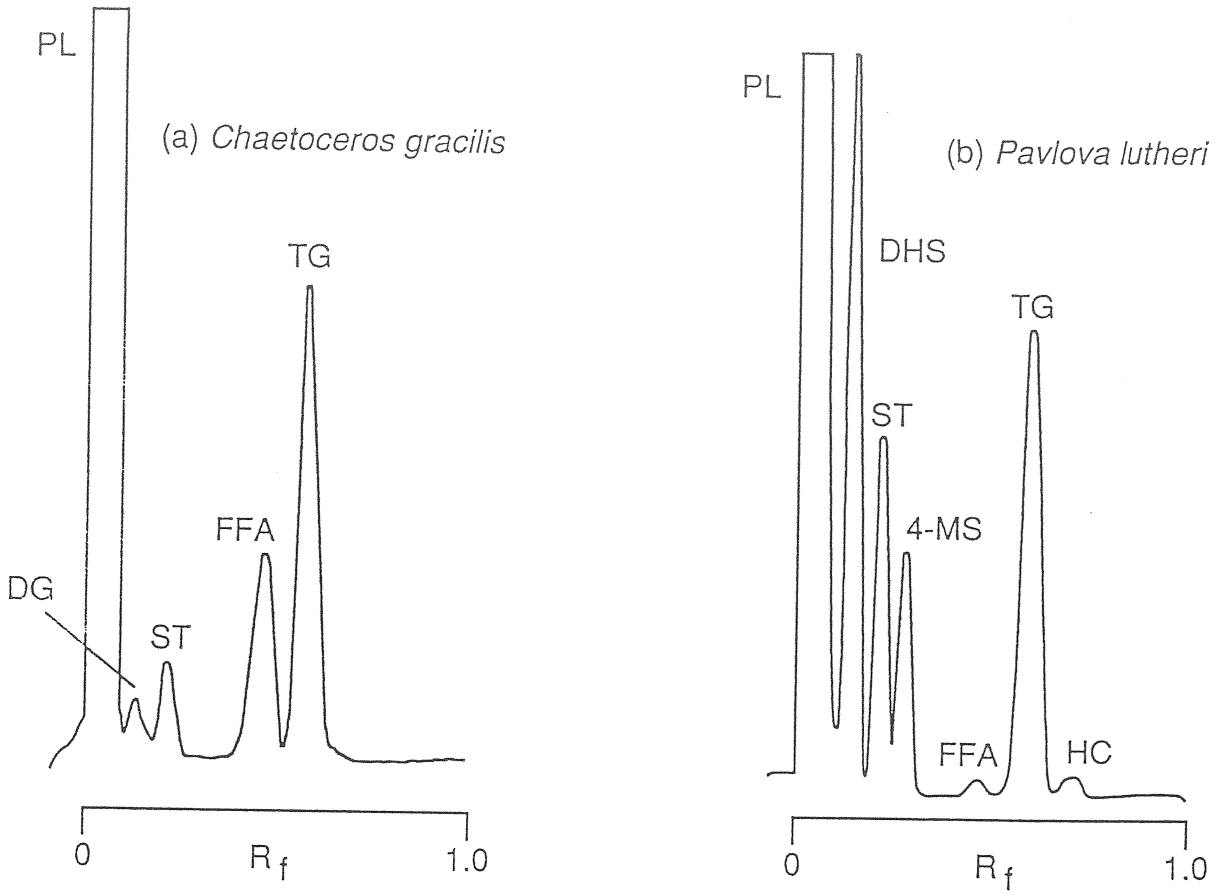


Figure 3. Typical Iatroscan TLC–FID chromatograms of lipids in microalgae. PL: polar lipids including phospholipids; DG: diacylglycerols; DHS: dihydroxy sterols; ST: sterols; 4–MS: 4–methyl sterols; FFA: free fatty acids; TG: triacylglycerols; HC: hydrocarbons.

4.5 Analysis by capillary gas chromatography

The complexity of marine fatty acid mixtures and the presence of double bond positional isomers makes it essential that the analysis system has maximum resolution and sensitivity. We used both polar and non-polar capillary columns to confirm identifications, and to enable poorly resolved peaks to be accurately quantified. Cooled on-column injection is also preferred since this minimizes losses of thermally-labile compounds such as polyunsaturated fatty acids (PUFA).

Fatty acid methyl esters (FAME) were analysed on a nonpolar methyl silicone fused-silica capillary column (HP1: 50 m x 0.32 mm i.d.) or one of several polar bonded-phase carbowax fused-silica capillary column (e.g. BP-20, BP-X70: 50 m x 0.32 mm i.d.). Carbowax columns are now widely used for these analyses (e.g. Napolitano *et al.*, 1988), and these are recommended for use in the Official A.O.C.S. method for determining fatty acids in marine oils.

Each fatty acid fraction was analysed with a Shimadzu 9A gas chromatograph equipped with an FID and cooled OCI-3 on-column injector (SGE, Australia). Samples were dissolved in chloroform to which known amounts of methylheptadecanoate (17:0) or *n*-docosane (n-C_{22}) were added as internal standards. Samples were injected at 45 ^{o}C onto a nonpolar methyl silicone fused-silica capillary column. After one minute, the oven temperature was raised to 120 ^{o}C at 30 ^{o}C per minute and then to 320 ^{o}C at 3 ^{o}C per minute. This final temperature was then maintained for 20 minutes. Hydrogen was used as the carrier gas. The detector temperature was 330 ^{o}C. A partial chromatogram showing a typical distribution of fatty acid methyl esters from an alga analysed on a non-polar capillary column is shown in Figure 4.

The fatty acid samples were further analysed with a Hewlett-Packard 4890 gas chromatograph equipped with an FID and cooled OCI-3 on-column injector and a polar 70% cyanopropyl siloxane (BP-X70) fused-silica capillary column (50 m x 0.25 mm i.d.) (SGE, Australia) or a polar Supelcowax™ 10 fused-silica column (60 m x 0.32 mm i.d.) (Supelco, Belleforte, PA) for earlier analyses. Samples were injected at 45°C and after 2 minutes the oven temperature was raised to 120°C at 30°C per minute and then to 240°C at 3°C per minute. The final temperature was maintained for 10 minutes. Hydrogen was used as the carrier gas. The detector temperature was 260°C.

TMS-derivatized nonsaponifiable lipids (NSL - "neutrals") fractions were analyzed with the Shimadzu GC or later in the project with a Varian High Temperature Series 5410 gas chromatograph with a Series 8100 autosampler and a septum-equipped programmable injector (SPI). A non-polar column was used with the same temperature program as above except that the second temperature ramp was 3° C min.$^{-1}$. The initial temperature of the SPI was 50 °C which was held for 0.15 min., then raised to 320° C at a rate of 150° C per min. and maintained for 5 min. Hydrogen was used as the carrier gases in both gas chromatographs, and both detector temperatures were 330°C.

Peak areas in chromatograms were quantified with DAPA software (DAPA Scientific Pty. Ltd., Kalamunda, Western Australia) and converted to concentrations using appropriate response and recovery factors. Compounds were identified from retention indices, coinjection with authentic standards and from mass spectra obtained by GC–MS.

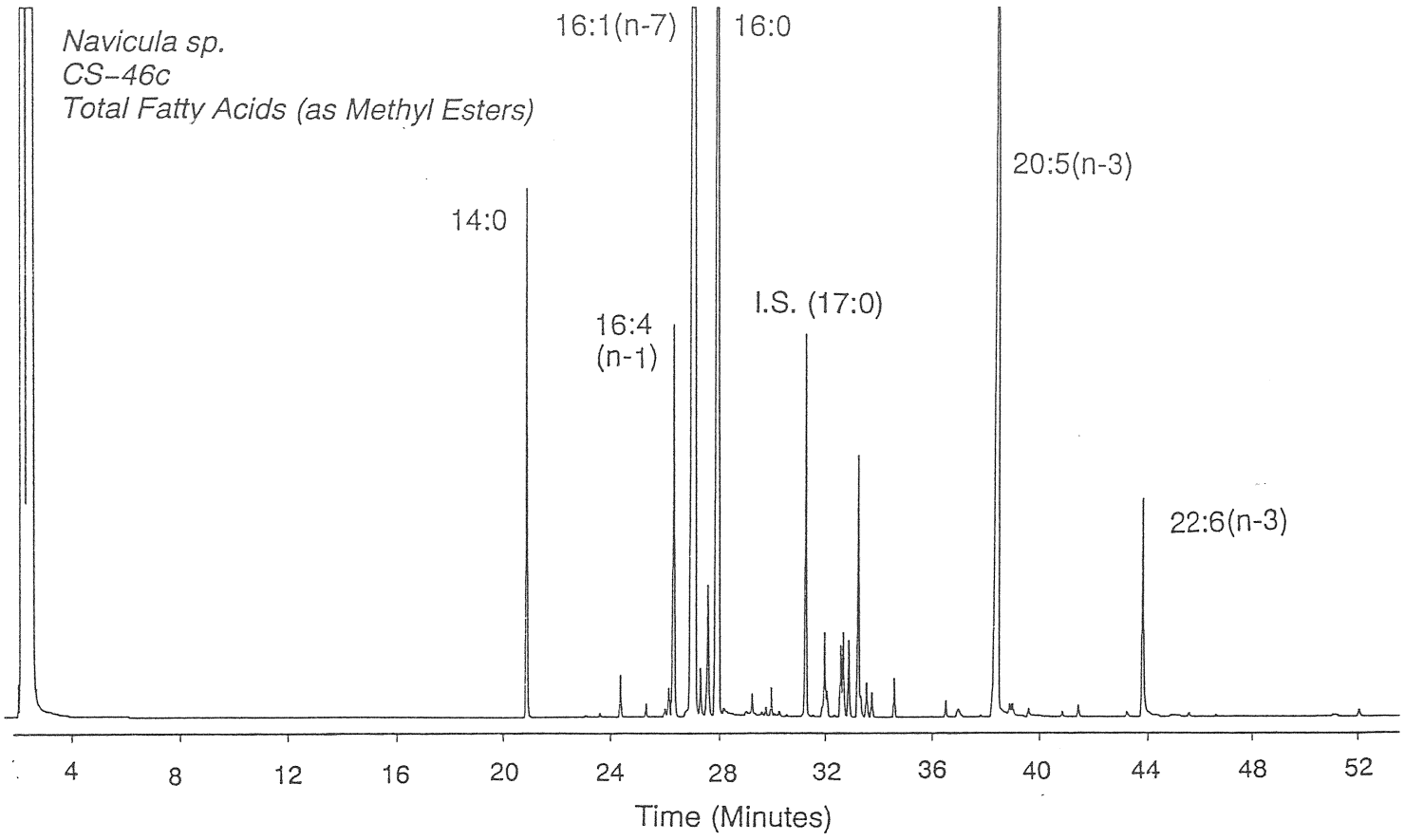


Figure 4. Capillary gas chromatogram showing distribution of total fatty acids (as methyl esters) in the marine diatom *Navicula* sp.. Note the high abundance of 16:0, 16:1(n–7) and 20:5(n–3) which is a common feature of diatom lipids.

4.6 Analysis of fatty acids, sterols and other lipids by gas chromatography–mass spectrometry (GC–MS).

Chemical structures were confirmed by comparison of mass spectra with those of authentic standards and with data reported in the literature. These GC–MS analyses were performed with a Hewlett Packard 5790 Mass Selective Detector (MSD) fitted with a direct capillary inlet. The nonpolar column, injector and chromatography conditions were similar to those described for the Shimadzu GC with the exception that helium was used as the carrier gas. Electron impact mass spectra were acquired and processed with an HP 59970A Computer Workstation. Typical MSD operating conditions were: electron multiplier 2000 volts; transfer line 310 ^{o}C; electron impact energy of 70 eV; 0.8 scans per second; mass range 40–650 dalton.

5. Results

5.1 Surveys of fatty acids in microalgae (Objectives 1 and 2)

Summary

As part of this project we carried out extensive studies of the lipid classes, fatty acids, sterols and other compounds in 56 microalgal strains including green algae, diatoms, prymnesiophytes, eustigmatophytes, cryptomonads and a red alga obtained from the CSIRO Algal Culture Collection. Table 1 provides a complete list of the species studied.

Our data show that there is a large variation in the content and composition of lipid classes between algal species. In some species triacylglycerols are abundant, while in others polar lipids such as phospholipids are the predominant lipid class found. Sterol contents were also highly variable between species.

The amount and distribution of essential polyunsaturated fatty acids varied greatly between algal classes and we are now in a position to make reasonable generalisations about what is a "typical" composition for all of the classes of algae used by the mariculture industry. Details of the compositions found in each of the major algal groups are provided in the following sections.

Figure 5 summarizes the average abundances of the 3 most important PUFA 20:4(n−6), 20:5(n−3) and 22:6(n−3) in the main classes of microalgae used in mariculture drawn from our data and information in the literature. The fatty acid contents show systematic differences according to taxonomic position, although it must be noted that there are a few examples of major differences between species from the same algal class (e.g. Volkman *et al.*, 1981; Pillsbury, 1985; Ben−Amotz *et al.*, 1987).

Diatoms in general contain high contents of 16:0, 16:1 and 20:5(n−3) with low abundances of 22:6(n−3), whereas chlorophytes contain very low amounts of 20:5(n−3) or 22:6(n−3) and instead have very high contents of C_{18} PUFA. Species from the Prymnesiophyceae are often excellent sources of 20:5(n−3) or 22:6(n−3) or both PUFA. The proportions of each fatty acid can also be changed by altering the culture conditions or by harvesting at different growth stages (see later).

A detailed discussion for each of the major algal classes follows.

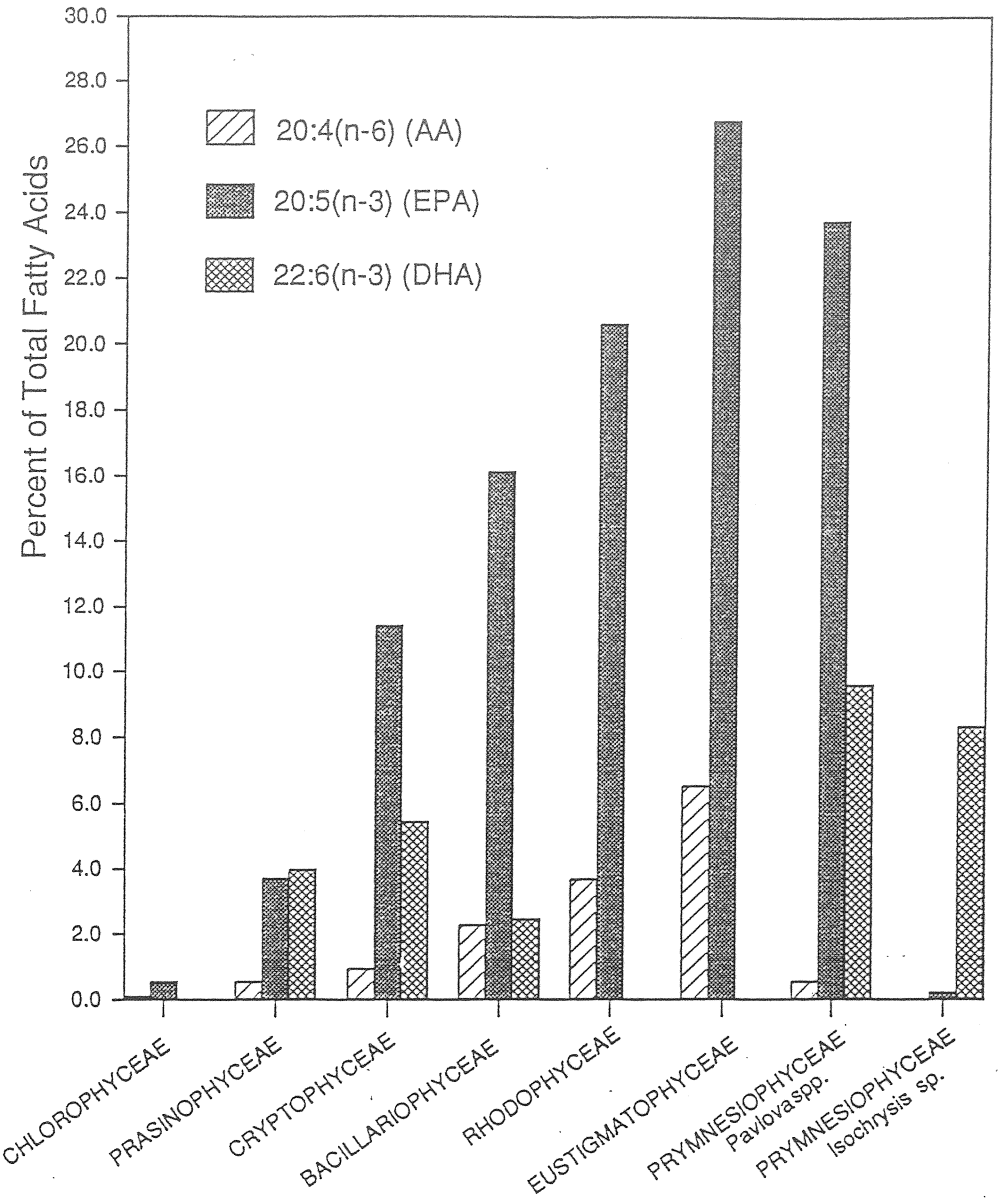


Figure 5. Summary of the average percentage compositions of long-chain polyunsaturated fatty acids 20:4(n–6), 20:5(n–3) and 22:6(n–3) in the total fatty acids of microalgal classes commonly used in mariculture

Table 1. Microalgae from the CSIRO Algal Culture Collection that have been cultured for lipid and fatty acid analysis.

Species name	CSIRO code	Species name	CSIRO code
	BACILLARIOPHYCEAE (diatoms)		
Chaetoceros calcitrans	CS-178	*Chaetoceros gracilis*	CS-176
Skeletonema costatum	CS-181	*Thalassiosira pseudonana*	CS-173
Nitzschia closterium	CS-5c	*Skeletonema* sp.	CS-252
Navicula sp.	CS-46c	*Coscinodiscus* sp.	CS-151
Haslea ostrearia	CS-250	*Thalassiosira stellaris*	CS-16
Amphora sp.	CS-10c	*Cylindrotheca fusiformis*	CS-13c
Amphiprora hyalina	CS-28	*Rhizosolenia setigera*	CS-62
Fragilaria pinnata	CS-121	*Thalassiosira rotula*	CS-77
Thalassionema nitzschioides	CS-146	*Thalassiothrix heteromorpha*	CS-132-8
	CHLOROPHYCEAE (green algae)		
Dunaliella tertiolecta	CS-175	*Nannochloris atomus*	CS-183
Stichococcus sp.	CS-92	*Chlorella* sp.	CS-195
Chlorella protothecoides	CS-41	*Chlorella* sp.	CS-247
	PRASINOPHYCEAE (green algae)		
Tetraselmis suecica	CS-187	*Tetraselmis chui*	CS-26
Pyramimonas cordata	CS-140	*Micromonas pusilla*	CS-98
Micromonas aff. pusilla	CS-170	Unidentified sp.	CS-126
Pycnococcus provasolii	CS-185		
	PRYMNESIOPHYCEAE (prymnesiophytes, golden brown flagellates)		
Isochrysis sp. (Tahitian)	CS-177	*Pavlova lutheri*	CS-182
Pavlova sp.	CS-50	*Pavlova salina*	CS-49
Pavlova sp.	CS-63	*Pavlova pinguis*	CS-286
Emiliania huxleyi	CS-PL06	*Emiliania huxleyi*	CS-57
Pseudoisochrysis paradoxa	CS-186	*Imantonia rotunda*	CS-194
Dicrateria inornata	CS-254	*Diacronema vikianum*	CS-266
Gephyrocapsa oceanica	CS-JB02		
	EUSTIGMATOPHYCEAE (yellow-green algae)		
Nannochloropsis oculata	CS-216	*Nannochloropsis salina*	CS-190
Nannochloropsis sp.	CS-246	*Nannochloropsis oculata*	CS-179
	CRYPTOPHYCEAE (cryptomonads)		
Chroomonas salina	CS-174	Unidentified sp.	CS-(PCL)
Chroomonas placoidea	CS-200	*Chroomonas* sp.	CS-24
Unidentified sp.	CS-(Qld)	*Rhodomonas* sp.	CS-215
Cryptomonas maculata	CS-85		
	RHODOPHYCEAE (red algae)		
Rhodosorus sp.	CS-210		

5.1.1 Lipids of diatoms

Summary

The lipids and fatty acids of 19 diatoms were studied in detail, which is possibly the most extensive study of this class of algae yet carried out by any research laboratory. In many other species of microalgae, triacylglycerols (storage lipids) are particularly abundant only when the algae are grown to stationary phase, but these lipids have been found to accumulate in the diatoms even in logarithmic phase making this group of microalgae a particularly rich source of lipids. A paper describing the data for 14 strains has been published (Dunstan *et al.*, 1994). Many of these species are new Australian isolates which had not previously been analysed.

Most of the diatoms examined were found to contain very high concentrations of 20:5(n–3) PUFA which appears to be a common feature of this class of algae. Some of these species are regularly requested from the CSIRO culture collection as they have been proven to be valuable feeds for juvenile abalone; others are important food for a variety of larval animals. Very few diatoms contained significant contents of 22:6(n–3), probably because a significant proportion is decarboxylated to the 21:6 alkene.

A large variety of sterol distributions was observed which contrasts with earlier conclusions that diatoms had high contents of either 24–methylenecholesterol, 24–methylcholesta–5,22E–dien–3β–ol or cholesterol. These sterols were certainly abundant in some species, but other sterols predominated in several species. For example, 24–ethylcholesta–5,22E–dien–3β–ol was found in several species from the order Thalassiophysale. This observation might prove to be useful for taxonomic assignment (Barrett *et al.*, 1995). One diatom (*Navicula* sp.) also had significant amounts of 4–methyl sterols including 4,23,24–trimethyl–5α–cholest–22E–en–3β–ol which is usually associated with dinoflagellates (Volkman *et al.*, 1993a).

Several diatoms contained unusual lipids. *Rhizosolenia setigera* contained novel C_{30} isoprenoid alkenes (Volkman *et al.*, 1994a). A closely related species *Rhizosolenia aff. chunii* is thought to be the cause of the bitter taste in mussels from Port Phillip Bay which have fed on the alga. These unusual compounds may prove to be useful markers for identifying marine animals which have fed on *Rhizosolenia*.

Microalgae from the class Bacillariophyceae (diatoms) are important in marine food webs. There several thousand species and, according to some taxonomic schemes, there are 87 genera and (Sournia *et al.*, 1991). Some species which grow well in mass culture have been used successfully as microalgal feedstocks for larval animals reared intensively (Enright *et al.*, 1986a). Since the primary storage material in diatoms is lipid, they are of potential value to the biotechnology industry for the production of lipids and in particular, polyunsaturated fatty acids. Relative to the large number of species within this group, very few species have been examined for lipid class and fatty acid composition (e.g. Ackman *et al.*, 1968; Chuecas and Riley, 1969; De Mort *et al.*, 1972; Orcutt and Patterson, 1975), and only a few comparative studies have been performed using high resolution analytical techniques (e.g. Volkman *et al.*, 1989).

Diatoms generally contain high concentrations of the (n–3) PUFA 20:5(n–3) and smaller proportions of 22:6(n–3) (e.g. De Mort *et al.*, 1972; Orcutt and Patterson, 1975; Watanabe *et al.*, 1983) which suggests that these microalgae may be a suitable source of PUFA for the supplementation of mariculture feedstocks. Some species of diatom have also been reported to contain significant proportions of the (n–6) PUFA arachidonic acid [20:4(n–6)] (e.g. Volkman *et al.*, 1979; Watanabe *et al.*, 1983).

The lipid class and total fatty acid compositions of fourteen species of diatom studied by us are shown in Table 2. The major lipid class in these microalgae was polar lipid (50.1–90.8%), although in some species triacylglycerols (0.3–47.7%) and free fatty acids (0–29.2%) were also abundant in the logarithmic phase cultures. Triacylglycerols (36.6 – 50.3%) and polar lipids (43.4 – 59.3%) comprised over 93% of the total lipids in the species studied (Table 2). Natural populations of diatoms have been found to have similar patterns of lipid abundances, although it should be noted that the abundance of triacylglycerols can be particularly variable and it is dependent on growth phase and nutrient status.

The major fatty acids in most species were 14:0, 16:0, 16:1(n–7) and 20:5(n–3). The polyunsaturated fatty acids 16:2(n–4), 16:3(n–4), 16:4(n–1), 18:4(n–3) and 22:6(n–3) also comprised a significant proportion (>4% each) of the total fatty acids in some species. In contrast, the main fatty acids in dinoflagellates are 16:0, C_{18} PUFA and 22:6(n–3) (e.g. Nichols *et al.*, 1984), and with few exceptions the characteristic fatty acid composition of diatoms is readily distinguishable from those of other classes of microalgae.

Small differences in lipid composition are apparent within the diatoms. For example, the mean proportion of 22:6(n–3) was significantly higher in representatives from the order Centrales (4.9%) than the Pennales (1.6%), but there was no consistent difference in the proportions of the other fatty acids (Tables 3 and 4). Four of the pennate diatoms contained significant proportions of arachidonic acid, 20:4(n–6) (3.5–5.6%), but most species examined contained very low proportions of (n–6) polyunsaturated fatty acids (Table 4). Based on the lipid compositions, all of the diatom species studied are potentially

suitable sources of 20:5(n–3) and some of the other longer–chain polyunsaturated fatty acids.

4–Desmethyl sterols were comparatively minor compounds and represented 0.8–1.9% of the total lipids with 4–methyl sterols and alcohols each less than 0.7%. Total sterols comprised 0.11 – 0.60% dry wt. which is within the range of 0.06 – 0.57 (average 0.23%) reported by Orcutt and Patterson (1975) from a study of 11 diatoms. Total sterols are usually much more abundant in dinoflagellates (e.g. 1.5 and 3.1% dry wt: Nichols *et al.*, 1984) than in diatoms.

Common constituents of the saponified neutrals fraction of most diatoms included phytol (from hydrolysis of chlorophyll a) and the common hydrocarbons n–heneicosahexaene (n–$C_{21:6}$) and squalene. The decarboxylase enzyme which is thought to form the *n*–$C_{21:6}$ hydrocarbon from 22:6(n–3) PUFA in microalgae (Lee and Loeblich, 1971) may reduce the amounts of this "essential" PUFA. This appears to be particularly so in the case of diatoms. In spite of the reduced proportions of 22:6(n–3) due to decarboxylation, the diatoms still contained very high proportions of the other essential (n–3) PUFA, 20:5(n–3) (12.2–30.2%).

Navicula species are particularly common in marine environments. For example, Hendey (1974) recognized over 170 species of *Navicula* in waters off Great Britain. Most species are associated with benthic habitats and some show promise as abalone feeds. Detailed culture studies were carried out with one species which contained a novel C_{34} tetra–unsaturated alcohol and exhibited an unusual sterol distribution including 4–methyl sterols. A chromatogram showing the fatty acids in this species is shown in Figure 4. The alga was cultured 3 times and in each case the lipid distributions were broadly similar apart from small quantitative differences thought to be due to the effects of differing culture conditions and culture age, and genetic differences between clones. For example, the weight per cell varied from 43 to 170 pg/cell. Some of the changes noted may be due to culture conditions (e.g. using blue light in the second and third experiments), but the data suggest that the effects of this change were relatively minor. The two replicate cultures gave similar concentrations per cell for most lipid classes except that one had a slightly higher abundance of triacylglycerols and polar lipids.

Two species of marine diatoms, *Haslea ostrearia* and *Rhizosolenia setigera* were shown to produce unusual C_{25} and C_{30} highly branched isoprenoid alkenes. This represents the first isolation and identification of these hydrocarbons from cultures of marine microalgae. Without excluding the possibility that other classes of algae may produce these isoprenoid hydrocarbons, our data show clearly that diatoms are likely to be a major source in present–day marine environments. A suite of C_{30} HBI alkenes have been found in mussels and seawater particulate matter in Port Phillip Bay, southeastern Australia (A. P. Murray and J. K. Volkman, unpublished data), which show a similar distribution to those found in *R. setigera*. This study of mussels and seawater particles was performed at the time of a bloom of *Rhizosolenia aff. chunii* in Port Phillip Bay which rendered mussels and

shellfish in the area bitter-tasting and hence unpalatable for seven months (Parry *et al.*, 1989). This strongly suggests that the source of the alkenes found was *R. chunii*, the slightly different hydrocarbons distribution to that found in *R. setigera* would probably be due to inter-species variations in the genus *Rhizosolenia*. Cultures of *R. chunii* are not yet available to test this hypothesis.

It is clear that only a small proportion of diatoms make these unusual compounds. The two species found here belong to different Orders (*Haslea* is in Pennales while *Rhizosolenia* is in Centrales), but other species from these Orders do not contain HBI alkenes (Table 1). The genus *Haslea* contains at least 9 species, some formerly of the genus *Navicula*, which have been isolated from both temperate and tropical marine environments (Simonsen, 1974; Ricard, 1987a). *Haslea ostrearia* is common in coastal areas and it produces an unusual pigment called marennine which is responsible for the blue-green colouration of the gills found in oysters grown in sea basins (Neuville and Daste, 1973 and 1978; Robert *et al.*, 1975; Robert, 1986). *Rhizosolenia* is a common genus in coastal and oceanic marine waters world-wide, with several species found in Antarctica and some in the Arctic regions (e.g. Priddle *et al.*, 1990). There are about sixty marine and only five freshwater species known (Ricard, 1987b). In some cases, the taxonomy, even to genus level, is uncertain (Priddle and Fryxell, 1985). Sundström (1986) has proposed a revision of the genus *Rhizosolenia*, and recommended that some species, including *R. setigera*, should be transferred to other genera.

Diatoms such as those examined in this work could be a useful source of sterols in the diets of those crustaceans and gastropods which can dealkylate some C_{28} and higher sterols to cholesterol, and also in the diets of bivalves which do not appear to need high concentrations of cholesterol. Full details of the sterol distributions found are given in Barrett *et al.* (1995). Species in which cholesterol is in high or moderate abundance such as *C. fusiformis, T. nitzschioides,* and *Skeletonema sp.*, or, for example, the NRAC-Skel strain of *Skeletonema costatum*, and various strains of *Chaetoceros* analyzed by Tsitsa-Tzardis *et al.* (1993) could be useful direct sources of cholesterol for farmed oysters (Tsitsa-Tzardis *et al.* 1993) and particularly for crustaceans and gastropods which require cholesterol as their main sterol. Species such as *R. setigera* and *N. closterium* (a favored abalone food) containing mainly cholesta-5,24-dien-3β-ol (desmosterol) might also be useful feeds as this sterol is the immediate precursor to cholesterol in the conversion process (Dadd 1982, Teshima 1982 and references cited therein). Of course, species of other algal groups containing high levels of cholesterol, such as eustigmatophytes where it is the dominant sterol (Volkman *et al.* 1992), are also direct sources of cholesterol for marine animals, and indeed *Nannochloropsis oculata* (also known as "marine Chlorella") is a widely used mariculture feed. Other strains of *Nannochloropsis* we examined also contained high contents of cholesterol (over 75% of the total sterols; see later).

Table 2. Diatom species examined, growth temperatures, growth media, total cellular lipid content (pg cell^{-1}) and percentage composition (%) of individual lipid classes.

Species	CSIRO Strain	Culture Temp.°	Media.	Total lipids (pg cell^{-1})	*Lipid class as a percentage of total lipids** PL	PG	DG&ST	FFA	TG	HC
CENTRALES										
Coscinodiscus sp.	CS–151	20	f/2	1196	84.3	–.	2.3	–	11.9	1.5
Rhizosolenia setigera	CS–62	20	f/2	114	72.4	4.1	3.1	9.4	7.1	3.9
Skeletonema costatum	CS–181	20	f/2	10	63.6	13.3	0.7	17.4	4.5	0.3
Skeletonema sp.	CS–252	25	f/2	3.2	81.9	6.2	1.7	7.9	1.3	0.6
Thalassiosira stellaris	CS–16	20	G	30	84.7	–	2.1	2.1	9.3	1.8
PENNALES										
Amphiprora hyalina	CS–28 §	20	f/2	16	81.4	6.3	1.4	6.8	2.1	2.0
Amphora sp.	CS–10 §	20	f/2	20	92.4	tr¶	0.9	2.1	3.1	1.5
Cylindrotheca fusiformis	CS–13 §	20	f/2	8.2	90.7	tr	2.1	2.9	2.5	1.8
Fragilaria pinnata	CS–121	25	f/2	5.4	89.1	–	3.7	0.9	5.0	1.3
Haslea ostrearia	CS–250–2	20	f/2	67	53.9	4.6	1.7	14.7	14.5	10.1
Navicula sp.	CS–46 §	20	f/2	11	50.1	tr	0.1	–	47.7	2.0
Nitzschia closterium	CS–5 §	20	f/2	9.7	89.7	8.4	0.3	1.0	0.3	0.3
Thalassionema nitzschioides	CS–146	28	f/2	11**	85.2	–	1.8	5.1	2.5	1.4
Thalassiothrix heteromorpha	CS–132–8	20	f/2	45	59.4	3.2	3.6	25.9	5.9	2.0

* PL = polar lipids and chlorophylls; PG = unidentified peak includes some pigments; DG&ST = diacylglycerols and sterols; FFA = free fatty acids; TG = triacylglycerols; HC = hydrocarbons; total lipid includes minor contributions from alcohols.

. see Jeffrey (1980) for details; . not detected; § axenic; ¶ trace less than 0.1%

** 4% of total lipid was an unidentified peak eluting between free fatty acids and triacylglycerols on TLC–FID.

Table 3. Relative proportions (%) of individual fatty acids and cellular content (pg cell^{-1}) of total fatty acids from five species of centric diatom (Centrales)

Species	*Coscinodiscus* sp.	*Rhizosolenia setigera*	*Skeletonema costatum*	*Skeletonema* sp.	*Thalassiosira stellaris*
Strain	CS–151	CS–62	CS–181	CS–252	CS–16
Saturated fatty acids					
14:0	10.5	17.6	11.9	19.6	11.0
15:0	0.4	0.4	0.3	0.5	0.5
16:0	16.5	16.4	5.1	3.9	8.3
18:0	0.4	1.3	0.1	0.3	0.4
24:0	–	0.1	–	0.1	0.1
Subtotal*	28.0	36.6	17.5	24.5	20.3
Monounsaturated fatty acids					
16:1(n–7)	14.6	17.7	19.1	19.8	17.1
16:1(n–5)	0.2	0.3	0.3	0.6	0.2
16:1(n–13)*t*	1.3	0.9	0.7	0.4	1.0
18:1(n–9)	1.0	0.4	0.3	0.3	0.4
18:1(n–7)	3.2	7.6	0.2	2.2	1.9
22:1	tr	–	–	0.2	–
24:1	–	–	–	0.5	0.8
Subtotal*	21.0	27.2	20.6	23.9	21.9
Polyunsaturated fatty acids					
16:2(n–7)	0.6	1.4	1.0	4.1	2.2
16:2(n–4)	2.1	1.8	0.9	13.3	4.4
16:3(n–4)	4.9	1.0	16.9	7.8	14.9
16:4(n–1)	4.2	3.5	8.1	5.6	1.1
18:2(n–6)	1.6	1.9	1.4	0.5	0.5
18:3(n–6)	0.8	0.5	–	0.2	0.3
18:3(n–3)	0.6	0.6	0.8	0.4	0.2
18:4(n–3)	3.5	1.7	2.8	1.0	5.2
20:3(n–6)	0.1	0.3	–	0.1	0.1
20:4(n–6)	1.1	1.0	–	tr	0.1
20:4(n–3)	0.5	–	0.2	0.1	0.1
20:5(n–3)	26.0	17.5	26.1	18.3	25.3
22:4(n–6)	–	–	–	–	–
22:5(n–6)	0.7	tr	–	–	0.6
22:5(n–3)	0.1	–	–	tr	tr
22:6(n–3)	4.6	6.1	4.7	4.2	4.8
Subtotal*	51.0	36.2	62.0	51.6	57.8
Total	100.0	100.0	100.0	100.0	100.0
Cellular content of fatty acids					
(pg cell^{-1})	900	52	5.6	1.6	19

*Subtotals include some minor fatty acids not listed (<0.8% each) ; nd: not detected; tr: trace; less than 0.1%.

Table 4. Relative proportions (%) of individual fatty acids and cellular content (pg cell^{-1}) of total fatty acids from nine species of pennate diatom (Pennales)

Species	*Amphiprora hyalina*	*Amphora sp.*	*Cylindrotheca fusiformis*	*Fragilaria pinnata*	*Haslea ostrearia*	*Navicula sp.*	*Nitzschia closterium*	*Thalassionema nitzschioides*	*Thalassiothrix heteromorpha*
Strain	CS-28	CS-10	CS-13	CS-121	CS-250-2	CS-46	CS-5	CS-146	CS-132-8
Saturated fatty acids									
14:0	7.1	6.0	8.7	1.4	8.6	4.9	8.2	23.3	28.9
15:0	0.3	0.4	0.6	0.4	0.4	0.4	0.4	0.4	0.3
16:0	10.7	14.6	20.0	25.2	20.4	21.3	7.2	6.6	2.9
18:0	0.2	0.3	0.4	0.8	0.8	0.4	0.1	0.5	0.6
24:0	0.8	0.7	0.3	0.1	0.2	0.1	–.	–	–
Subtotal*	19.2	21.9	30.1	28.2	33.2	27.2	15.9	30.9	32.7
Monounsaturated fatty acids									
16:1(n-7)	20.1	13.6	19.7	26.3	28.6	34.4	22.8	17.1	14.6
16:1(n-5)	0.1	0.1	0.6	0.1	0.3	0.4	0.8	0.3	0.1
16:1(n-13)*t*	0.7	1.4	0.1	0.2	0.1	1.5	1.4	0.7	0.5
18:1(n-9)	1.1	0.6	0.6	0.6	0.9	0.4	0.1	0.3	0.7
18:1(n-7)	2.3	0.2	0.3	4.3	1.6	0.3	0.2	1.4	3.9
22:1	0.1	–	–	–	–	–	–	tr.	1.4
24:1	–	–	–	–	–	–	–	–	1.9
Subtotal*	25.0	16.4	21.4	31.6	31.7	37.0	25.4	20.3	23.7
Polyunsaturated fatty acids									
16:2(n-7)	2.1	2.0	1.1	0.8	1.1	0.2	0.9	3.9	1.9
16:2(n-4)	2.8	5.1	3.0	4.5	4.1	2.4	9.4	3.1	1.6
16:3(n-4)	9.5	21.6	5.0	4.8	10.6	4.1	9.8	14.1	3.2
16:4(n-1)	2.7	0.2	5.4	tr	0.3	0.2	3.2	tr	18.6
18:2(n-6)	1.4	0.8	1.5	0.8	0.5	2.9	0.5	0.6	1.4
18:3(n-6)	0.3	0.2	2.8	0.4	0.7	0.7	0.5	0.7	0.7
18:3(n-3)	0.5	0.2	0.1	0.1	1.0	0.2	–	0.1	0.1
18:4(n-3)	5.5	0.9	2.0	1.1	0.9	0.9	3.5	0.1	0.6
20:3(n-6)	0.1	0.1	0.5	0.6	tr	0.1	–	0.2	0.3
20:4(n-6)	0.8	0.5	4.9	5.6	0.2	0.5	4.0	3.5	2.2
20:4(n-3)	0.2	0.8	0.7	0.2	0.7	0.1	0.1	0.1	0.1
20:5(n-3)	30.0	30.2	20.3	20.7	12.2	21.0	24.2	25.2	12.9
22:4(n-6)	–	–	–	0.2	–	–	–	–	–
22:5(n-6)	tr	–	0.8	–	tr	–	1.1	0.1	0.2
22:5(n-3)	0.1	0.2	0.3	1.0	0.2	tr	tr	–	0.1
22:6(n-3)	1.9	0.7	1.1	–	3.3	2.6	2.4	1.0	1.6
Subtotal*	55.8	61.7	48.5	40.2	35.1	35.8	58.7	48.8	43.6
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cellular content of fatty acids									
(pg cell^{-1})	9.6	11	2.2	3.0	27	7.0	4.3	3.8	18

*Subtotals include some minor fatty acids not listed (<0.8% each)
nd: not detected; tr: trace; less than 0.1%.

5.1.2 Lipids of green algae.

Summary

A detailed study of the lipids in 10 species of green microalgae was carried out. This work confirmed the lack of essential polyunsaturated fatty acids in microalgae of the class Chlorophyceae such as *Chlorella* (Dunstan *et al.*, 1992), which may explain why many of these algae are poor feedstocks when used as single species diets for larval production. In contrast, species from the class Prasinophyceae such as *Tetraselmis* contain small amounts (~10%) of C_{20} and/or C_{22} PUFA and it is these microalgae which have been successfully used as mariculture feedstocks.

Green microalgae (Division Chlorophyta) from the classes Chlorophyceae and Prasinophyceae (= Micromonadophyceae) are common in the marine environment (e.g. Hallegraeff, 1981). Several of these species are widely used in the mariculture industry as food for invertebrate animals, but feeding trials have resulted in mixed degrees of success. Some species are reported as good foods, while others have resulted in poor growth and survival of the animal being cultured (reviewed by Brown *et al.*, 1989). Prasinophytes are presumed to occupy a primitive position in the Chlorophyta (Mattox and Stewart, 1984), and they have a wide geographical and geological range. They can be important constituents of the phytoplankton in present-day marine ecosystems (e.g. Hallegraeff, 1983; Guillard *et al.*, 1991), and they are potentially significant contributors of organic matter to sediments and seawater particulate organic matter.

In this report we provide details of the lipid (Table 5) and fatty acid (Table 6; Figure 6) compositions of four green algae from the Chlorophyceae (chlorophytes) and six from the Prasinophyceae (prasinophytes). Six species are new Australian isolates. The total lipid content was very similar in all species and fell in the range 89–167 pg per cell (Table 5). These were comprised almost entirely of polar lipids with triacylglycerols below detection in all species. Details of the amino acids, sugars and pigments in these same cultures are given by Brown and Jeffrey (1991).

Major fatty acids in the Chlorophyceae were 16:0, 16:1(n–13)*t*, 16:2(n–6), 16:3(n–3), 18:2(n–6) and 18:3(n–3). In the species examined, fatty acids with four or more double bonds were not detected, nor were any C_{20} and C_{22} fatty acids (Figure 6). A typical distribution showing the abundance of C_{16} and C_{18} PUFA in a tropical *Chlorella* isolate (CS–195) is shown in Figure 7. Major fatty acids in the Prasinophyceae were 16:0, 16:1(n–13)*t*, 18:1(n–7), 16:4(n–3), 18:4(n–3), with variable proportions of 18:3(n–3), 18:5(n–3), 20:5(n–3) and 22:6(n–3) (Figure 6). The distribution in *Tetraselmis chui* (CS–26) shown in

Figure 7 is typical of these species. Note the presence of 18:5(n–3) which is the first report of this unusual fatty acid as a constituent of green microalgae. Figure 7 also shows the distribution of PUFA found in a tropical isolate CS–126 initially thought to be a *Chlorella* species, but which is now believed to be a prasinophyte. The fatty acid distribution is certainly consistent with this view and provides a demonstration of the use of lipids in taxonomic investigations.

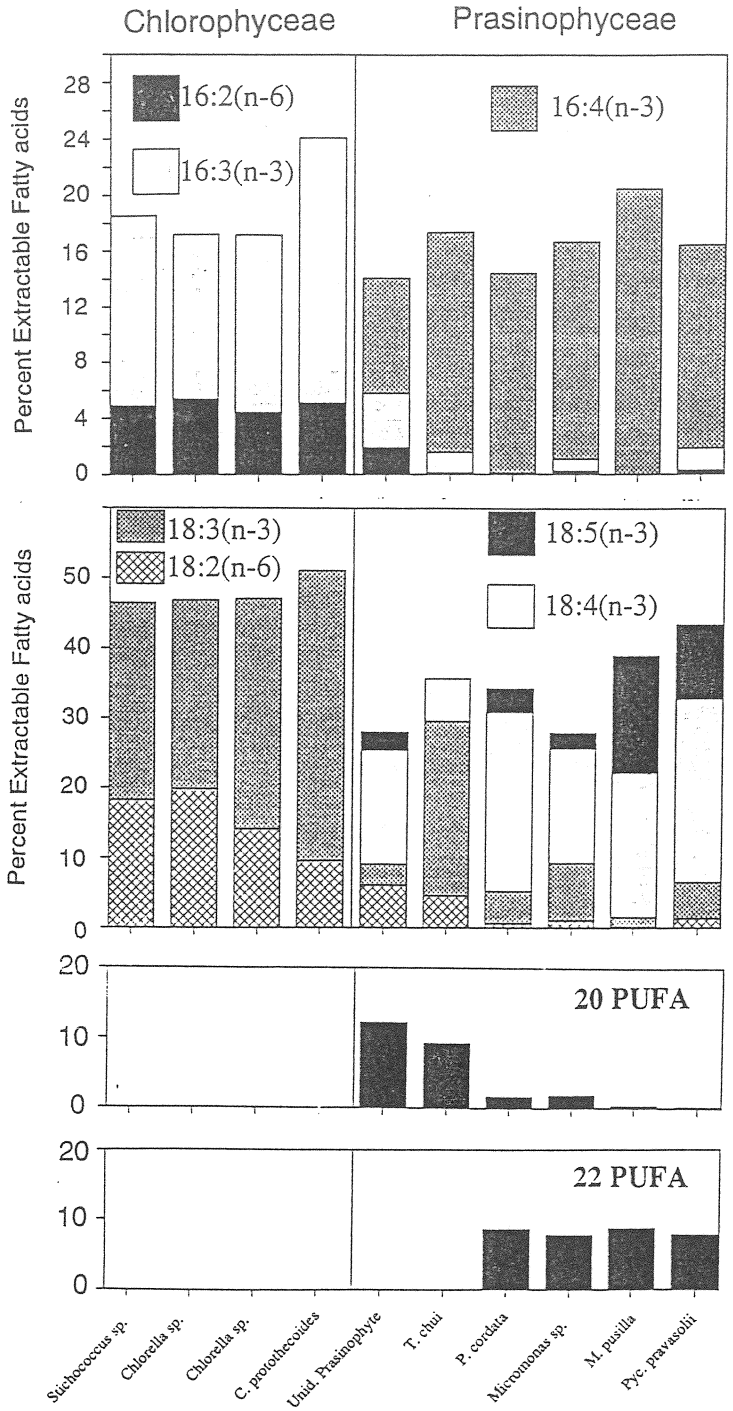


Figure 6. Relative abundances of C_{16}, C_{18}, C_{20} and C_{22} PUFA in green microalgae from the Chlorophyceae and Prasinophyceae. Note that the levels of C_{18} PUFA are lower in the prasinophytes, reflecting their chain–elongation to PUFA with 20 and 22 carbon atoms.

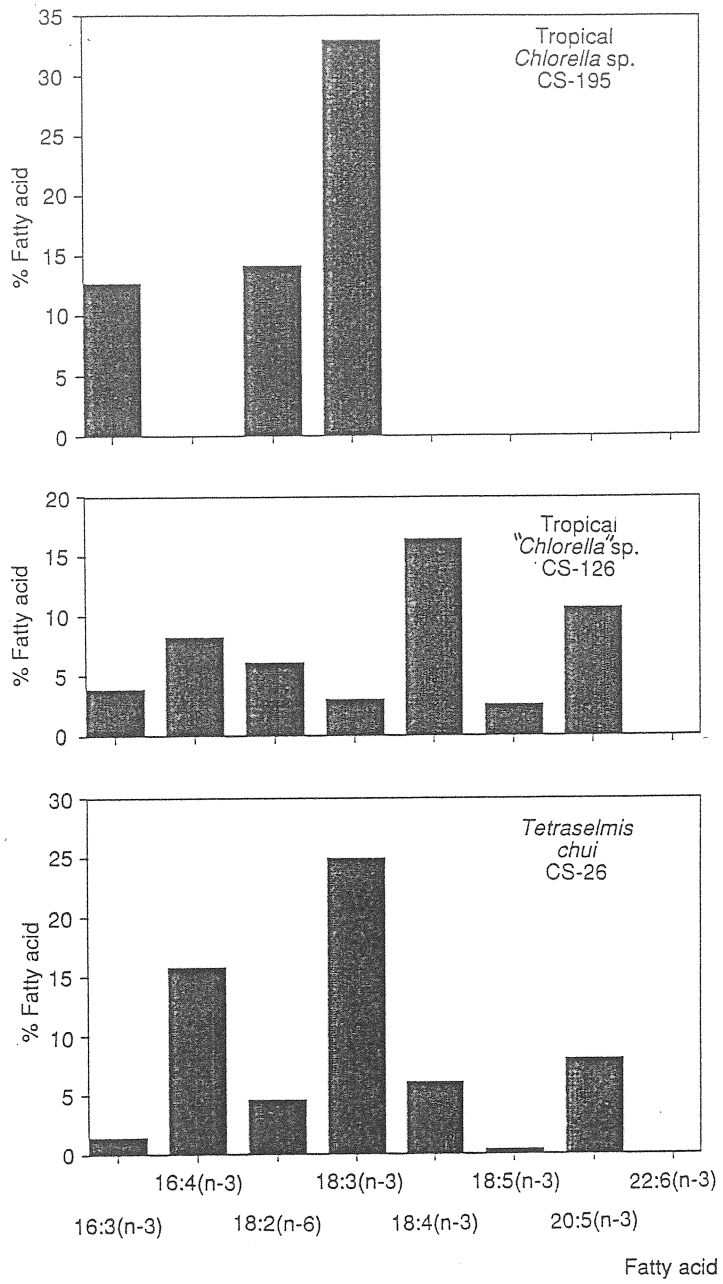


Figure 7. Percentage composition of major PUFA in 3 species of green microalgae. Note the lack of 20:5(n–3) and 22:6(n–3) in the tropical *Chlorella* sp. (CS–195). The presence of 20:5(n–3) in CS–126 is consistent with the identification of this strain as a prasinophyte.

Most species from the Chlorophyceae do not produce the essential long–chain polyunsaturated fatty acids 20:5(n–3) and 22:6(n–3) required by most marine animals, and thus are generally not suitable as a single species algal food in hatcheries. Rather they contain high concentrations of C_{16} and C_{18} polyunsaturated fatty acids (PUFA) with two and three double bonds. Note, however, that the chlorophyte *Nannochloris atomus* contains small amounts of some $C_{20} - C_{22}$ PUFA (Volkman *et al.*, 1989).

Previous studies have established that most marine animals maintain good growth in feeding trials when fed prasinophytes. Species of the prasinophyte genus *Tetraselmis* are easily cultured and widely used in the mariculture industry as food for larval and juvenile molluscs and crustaceans. In general terms, species of *Tetraselmis* have been found to be a better food than the chlorophytes, and may provide growth rates in young molluscs as high as those obtained using species of the prymnesiophyte *Isochrysis* (Walne, 1970; Enright *et al.*, 1986a). *Tetraselmis chui* is a valuable food for the prawn *Penaeus monodon* (Kontara *et al.*, 1986; Kurmaly *et al.*, 1989). *T. tetrathele* gave better results than "marine *Chlorella*" (= *Nannochloropsis oculata*) in feeding trials when fed to rotifers (*Brachionus plicatilus*) which were in turn fed to larvae of the red sea bream (Fukusho *et al.*, 1984) and flounder (Fukusho *et al.*, 1985). This is of interest because *N. oculata* contains a higher proportion of 20:5(n–3) than species of *Tetraselmis*, although it should be noted that because of the large size of the latter species, each cell contains significantly more of this PUFA (Table 6). It has been suggested that *Tetraselmis suecica* cannot be digested by *Crassostrea virginica* spat (Romberger and Epifanio, 1981) and other authors have reported low growth rates in juvenile oysters and clams when *Tetraselmis* species have been used as a food. One reason for these conflicting observations on bivalve growth rate may be the feed ration (Epifanio, 1979). Oyster spat may also find it difficult to utilize cells of this large size (mean cell volume 640 μm^3, Volkman *et al.*, 1989).

Larvae of *Crassostrea gigas* have been successfully reared on *Pyramimonas virginica* (Waldock and Nascimento, 1979), while juvenile clams (*Mercenaria mercenaria*) grew well when fed *Pyramimonas grossii* (Walne, 1970). The much smaller prasinophyte *Micromonas pusilla* only supported limited growth in juvenile clams (*Mercenaria mercenaria*) and oysters (*Crassostrea gigas* and *Ostrea edulis*), with better growth observed when the latter species was fed with *Micromonas squamata* (Walne, 1970). A possible explanation for these observations is that microalgae less than 1 μm in diameter are less easily taken by adult oysters (Brown and Jeffrey, 1991). It is interesting that *Pyramimonas cordata* contains an unusually high content of 22:5(n–3) (Table 6) which we have shown is important for abalone nutrition (unpublished data).

Several species of Chlorophyceae have been shown to be a poor food for species of oysters, mussels and clams. Davis and Guillard (1958) concluded that axenic cultures of three chlorophytes were of no food value to oyster (*C. virginica*) and clam (*Mercenaria mercenaria*) larvae. The low growth rates were attributed to toxic metabolites produced by the algae and to reduced digestibility due to thick cell walls. More likely, these effects may simply have been due to the poor nutritional qualities of the algae. Others have also noted

that chlorophytes are unsuitable foods for juvenile oysters (*O. edulis*) and clams (*M. mercenaria*) (Walne, 1970). Oysters showed limited growth when fed on *Dunaliella* (Langdon and Waldock, 1981; Enright *et al.*, 1986a), and turbot larvae had a low survival when fed rotifers which had been reared on *D. tertiolecta* (Scott and Middleton, 1979). *D. tertiolecta* also supported minimal growth in the queen conch, *Strombus gigas* (Pillsbury, 1985). Walne (1970) concluded that the chlorophyte *Nannochloris atomus* had a "reasonable nutritive value" for oyster spat, although juveniles of the clam *M. mercenaria* grew poorly when fed this species. *Nannochloris* sp. did not support growth in three species of bivalve larvae (Tan Tiu *et al.*, 1989). It should be noted that *Nannochloris atomus* is one of the few chlorophytes reported to contain long-chain PUFA (Volkman *et al.*, 1989).

The lack of long-chain PUFA 20:5(n-3) and 22:6(n-3) in chlorophytes, coupled with the extremely thick cell wall of many of the *Chlorella* species (which present a barrier to digestive enzymes), often makes these algae unsuitable as live food for filter feeding bivalves and their larvae. However, they have proven useful in the culture of crustaceans which can crush the cells with their hard mouth parts (reviewed by De Pauw *et al.*, 1984). The thick cell wall of species like *Chlorella* may be of an advantage in some cases since this enables them to be frozen for short periods without disrupting the cell (Stewart *et al.*, 1987). These microalgae have been used as a dry feed, but drying can result in loss of PUFA due to oxidation. Dried samples of *T. suecica* mixed with live microalgae have been successfully used as food for prawn larvae (*Penaeus vannamei*), but when dried algae were used alone this significantly reduced survival and metamorphosis of the prawn larvae (Biedenbach *et al.*, 1990). It is of interest that dried samples of *T. suecica* have recently been shown to exhibit antibacterial activity to prawn pathogens (Austin and Day, 1990).

Since most green algae are readily cultured and have favourable amino acid and carbohydrate profiles (Brown and Jeffrey, 1991), it has been suggested that these species would be suitable when mixed with microalgae known to contain essential long-chain PUFA (Chu and Dupuy, 1980; Webb and Chu, 1983). When *Tetraselmis suecica* was used in a mixed diet with microalgal species known to promote good growth in bivalves, better growth was attained than if *T. suecica* was used alone (Helm, 1977; Romberger and Epifanio 1981; Laing and Millican, 1986). Since many animals grow poorly when fed on single species diets of chlorophytes it is preferable to use them as part of mixed diets, or to use other microalgae with better nutritional features.

The sterols in the prasinophytes were studied in detail, but no attempt was made to characterize the complex mixtures of sterols found in the chlorophytes. Full details are reported in Volkman *et al.* (1994b). Our data show that prasinophytes contain small amounts of sterols; the distributions are quite simple and dominated by a few C_{28} and C_{29} Δ^5-unsaturated sterols. The C_{28} sterol 24-methylcholest-5-en-3β-ol may be a useful marker for these microalgae. Cholesterol contents were very low.

One unusual high molecular weight sterol was found in several species and finally identified as 24–ethylcholesta–5,28(29)–dien–3β,24–diol (saringosterol) by comparison with the same sterol isolated from dried samples of brown algae and literature data (Volkman *et al.*, 1994b). Saringosterol is a common constituent of dried macroscopic brown algae such as kelp, but it is not usually present in the fresh alga (Ikekawa *et al.*, 1966; 1968; Knights, 1970). This is the first report of its presence in microalgae, and its first detection in green algae. It is thought to be formed from the aerial oxidation of fucosterol, but in the present case the likely precursor is 28–isofucosterol. Other microalgae that we have analysed under the same conditions have not contained saringosterol even though suitable precursors were present. This suggests either that it is a natural product or that it is formed by extracellular enzymatic reactions when the cells are ruptured.

Table 5. Content (pg/cell) of solvent-extractable lipid classes in microalgae from the Chlorophyceae and Prasinophyceae determined by Iatroscan thin layer chromatography-flame ionization detection.

Species	CSIRO Culture Code	HC/WE	ST	PIG	PL	Content*	Concentration**
		pg.cell^{-1}				pg.cell^{-1}	mg/g dry wt
CHLOROPHYCEAE							
Chlorella protothecoides	CS-41	–	tr	–	3.8	3.8	141
Chlorella sp.	CS-247	–	tr	tr	0.74	0.74	104
Chlorella sp.	CS-195	–	tr	–	0.69	0.69	162
Stichococcus sp.	CS-92	–	tr	0.01	0.48	0.49	99.8
PRASINOPHYCEAE							
Pyramimonas cordata	CS-140	0.12	0.05	0.06	4.9	5.13	117
Tetraselmis chui	CS-26	–	0.24	–	22	22.2	102
Coccoid prasinophyte	CS-126	–	tr	–	0.68	0.68	143
Pycnococcus provasolii	CS-185	–	0.02	–	0.52	0.54	89.3
Micromonas pusilla	CS-98	0.01	0.01	0.01	0.36	0.39	101
Micromonas pusilla	CS-170	–	0.01	0.01	0.37	0.39	167

HC/WE, hydrocarbons and/or wax esters; ST, sterols; PIG unidentified peak including pigments; PL, polar lipids and chlorophylls; tr, less than 0.9%; Content* solvent extractable lipid content (pg/cell) determined by TLC-FID; Concentration** solvent extractable lipid concentration (mg/g dry wt.) determined by TLC-FID.

Table 6. Relative contents of major solvent-extractable fatty acids in microalgae from the Chlorophyceae and Prasinophyceae.

Class	CHLOROPHYCEAE			PRASINOPHYCEAE					
Species	*Chlorella protothecoides*	*Chlorella* sp.	*Chlorella* sp.	*Stichococcus* sp.	*Pyramimonas cordata*	*Tetraselmis chui*	Coccoid prasinophyte	*Pycnococcus provasolii*	*Micromonas pusilla*	*Micromonas pusilla*
Culture Code	CS-41	CS-247	CS-195	CS-92	CS-140	CS-26	CS-126	CS-185	CS-98	CS-170
Saturated Fatty Acids										
14:0	0.2	0.5	0.6	0.4	0.2	0.2	1.3	3.2	4.4	6.7
15:0	0.2	0.3	0.5	0.2	tr	–	1.0	0.1	0.0	0.3
16:0	15.5	23.6	21.8	24.5	13.4	19.9	26.3	20.2	13.6	20.6
18:0	0.5	0.9	0.8	0.5	1.3	0.3	0.5	0.5	1.0	1.8
24:0	–	0.7	0.3	0.4	–	0.1	tr	–	–	–
25:0	–	0.1	–	0.2	–	–	0.3	–	–	–
26:0	–	0.2	–	0.3	–	–	1.1	–	–	–
subtotal*	16.3	26.8	24.1	26.5	14.9	20.6	30.5	24.1	19.0	29.5
Monounsaturated Fatty Acids										
16:1(n-12)	–	–	–	–	–	–	–	–	1.9	–
16:1(n-10)	–	–	–	–	–	–	–	–	0.2	–
16:1(n-9)	0.3	0.7	1.0	0.4	1.0	0.3	0.5	0.8	0.1	0.2
16:1(n-7)	0.8	0.7	1.7	2.1	0.8	0.1	0.2	0.2	0.7	0.5
16:1(n-13)*t*	2.0	3.9	5.2	3.9	6.4	3.2	0.9	3.0	2.5	2.4
18:1(n-9)	3.5	2.2	2.4	0.8	0.2	6.8	0.7	2.2	0.3	1.0
18:1(n-7)	1.7	1.1	1.2	0.9	14.8	3.0	0.8	0.1	5.5	10.8
20:1(n-9)	–	–	–	–	–	2.5	–	–	tr	0.1
subtotal*	8.3	8.9	11.7	8.4	23.6	15.9	3.7	6.5	11.5	15.4

Table 6 continued

Class	CHLOROPHYCEAE				PRASINOPHYCEAE					
Species	*Chlorella protothecoides*	*Chlorella* sp.	*Chlorella* sp.	*Stichococcus* sp.	*Pyramimonas cordata*	*Tetraselmis chui*	Coccoid prasinophyte	*Pycnococcus provasolii*	*Micromonas pusilla*	*Micromonas pusilla*
Culture Code	CS-41	CS-247	CS-195	CS-92	CS-140	CS-26	CS-126	CS-185	CS-98	CS-170
Polyunsaturated Fatty Acids										
16:2(n-7)	–	–	–	–	–	–	–	–	0.2	–
16:2(n-6)	5.1	5.4	4.4	4.9	0.1	0.1	1.9	0.3	–	0.2
16:3(n-6)	–	–	–	–	–	–	2.9	0.1	–	–
16:3(n-3)	19.0	11.8	12.7	13.6	tr	1.5	3.9	1.6	–	0.9
16:4(n-3)	–	–	–	–	14.3	15.9	8.2	14.5	20.4	15.5
18:2(n-6)	9.6	19.8	14.1	18.3	0.7	4.6	6.1	1.4	0.1	1.1
18:3(n-6)	–	–	–	–	tr	0.4	5.6	0.7	tr	0.5
18:3(n-3)	41.5	27.0	32.9	28.2	4.6	25.2	3.0	5.2	1.4	8.1
18:4(n-3)	–	–	–	–	25.6	6.1	16.4	26.3	20.7	16.4
18:5(n-3)	–	–	–	–	3.4	–	2.6	10.6	16.7	2.2
20:3(n-3)	–	–	–	–	1.2	0.1	–	–	0.1	0.1
20:4(n-6)	–	–	–	–	–	0.6	1.4	–	–	–
20:4(n-3)	–	–	–	–	–	0.6	0.1	–	tr	0.1
20:5(n-3)	–	–	–	–	0.4	8.0	10.7	0.2	0.3	1.6
22:5(n-3)	–	–	–	–	4.1	–	–	0.4	0.3	0.3
22:6(n-3)	–	–	–	–	4.5	–	–	7.4	8.5	7.5
subtotal*	75.2	64.0	64.1	65.0	60.6	63.0	62.8	68.9	69.1	54.7
unidentified	0.2	0.3	0.1	0.2	0.9	0.2	3.0	0.6	0.4	0.4
TOTAL %	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Extractable Fatty Acids										
(pg/cell)	1.04	0.27	0.19	0.20	2.60	9.96	0.19	0.26	0.18	0.19
(mg/g dry wt.)	38.6	37.8	43.5	40.5	59.6	46.0	41.0	40.3	46.6	82.1
% of total FAME	61.6	94.4	98.2	97.4	95.6	97.9	84.0	–	97.5	93.9
% of solvent extractable lipids	27.4	36.3	26.9	40.6	50.9	45.1	28.7	45.1	46.1	49.2

* Subtotals include contributions from minor fatty acids not listed.; tr denotes trace amounts detected (less than 0.5%).

5.1.3 Lipids of Eustigmatophytes

Summary

In 1993, we published detailed biochemical analyses of the yellow–green eustigmatophytes which showed that these microalgae are excellent sources of the PUFA 20:5(n–3) (Volkman *et al.*, 1993b). This was not widely known by the mariculture industry at the time due to misnaming of one of the commonly used species from Japan as "marine *Chlorella*". This alga is still widely used in mariculture, and our work showed that it contains an unusually high concentration of 20:5(n–3) (Volkman *et al.*, 1993b).

The gross biochemical compositions of two strains of the marine eustigmatophyte *Nannochloropsis oculata* (CS–216 and CS–179), *Nannochloropsis salina* (CS–190) and an un–named tropical coccoid eustigmatophyte from tropical Australian waters were quite similar. Small quantitative differences were noted, probably reflecting differences in culture conditions. The distributions of individual lipid classes also matched closely, apart from an elevated content of triacylglycerols in *N. salina*. The compositional data for fatty acids, amino acids, sugars and pigments combined with the presence of cholesterol, unusual C_{30}–C_{32} 1,15–alkyl diols and C_{30}–C_{32} mono– and diunsaturated n–alcohols are sufficiently distinctive to be used as chemotaxonomic markers for this class of algae (Volkman *et al.*, 1992a). The nutritional significance of the alkyl diols is not known, but they do not seem to be associated with any detrimental effects.

High concentrations of the essential fatty acid 20:5(n–3) combined with good quality protein and carbohydrate and presence of cholesterol and vitamin C make these easily cultured microalgae very useful live diets for marine animals in mariculture. Additional information on the lipids in large scale cultures of *Nannochloropsis oculata* is given in Section 5.2.1.

The class Eustigmatophyceae was proposed by Hibberd and Leedale (1970, 1971, 1972) to accommodate certain species of yellow–green microalgae that had previously been assigned to the class Xanthophyceae. These two algal classes are thought to have diverged from the brown algal line (Chromophyta) more recently than the dinoflagellates and raphidophytes, but before the emergence of prymnesiophytes and diatoms (Taylor 1987). *Nannochloropsis oculata* was originally named *Nannochloris oculata* Droop, and *Nannochloropsis salina* was formerly known as *Monallantus salina* Bourrelly (Berland *et*

al. 1970, Antia *et al.* 1975, Hibberd 1981). Eustigmatophytes can be either unicellular or filamentous, and the coccoid forms many bear a superficial resemblance to some green algae, both in color and cell morphology, when examined under light microscopy. For this reason, strain CS–179 was previously and incorrectly referred to as "marine" *Chlorella* (Chlorophyceae), but Maruyama *et al.* (1986) showed from its cell ultrastructure and fatty acid and pigment compositions that this alga is identical with the previously described *Nannochloropsis oculata*.

Nannochloropsis oculata has been used as a live algal feedstock in mariculture for many years (e.g. Watanabe *et al.* 1983), and it grows well under mass culture. It contains very high concentrations of the polyunsaturated fatty acid 20:5(n–3). We undertook a detailed examination of the gross biochemical composition of other coccoid algae from the Eustigmatophyceae. The species studied were *Nannochloropsis salina* (strain CS–190) from Scotland, two strains of *Nannochloropsis oculata* (CS–179 and CS–216) from Japan and an unnamed eustigmatophyte (CS–246) isolated from Queensland waters which appears to be closely related to *N. oculata*. Amino acids, sugars, fatty acids and pigments were analyzed to determine which features of the biochemical composition might prove to be characteristic of this unusual group of microalgae, and to assess their potential as feedstocks for mariculture and biotechnology.

Gross compositional features were similar in the species studied (Table 7). Total carbohydrate ranged from 5.2% (*N. oculata* CS–179) to 8.9% (*N. salina*) of cell dry weight. Polysaccharide comprised from 74% (*N. oculata* CS–179) to 88% (CS–246) of this total. Glucose was the principal polysaccharide sugar (45.2% – 66.2% of total sugars). Other sugars included fucose, galactose, mannose, rhamnose, ribose and xylose (2.0–14.0%): Arabinose was a minor constituent in all species (0.6–1.7%). Protein varied from 17.8% (*N. salina*) to 22.1% (*N. oculata* CS–216) of the cell dry weight. The major amino acids were arginine, glutamate and aspartate (7.2–10.4% of total amino acids), with methionine, cystine, histidine, tryptophan, hydroxy–proline, ornithine and γ–aminobutyric acid (GABA) much less abundant (0.03–2.6%).

Lipid content ranged from 8.2% (*N. oculata* CS–216) to 16.9% (*N. salina*) of cell dry weight, the latter value reflecting enhanced concentrations of triacylglycerols in *N. salina*. The major fatty acids were palmitic acid (16:0), palmitoleic acid [16:1(n–7)] and eicosapentaenoic acid [20:5(n–3)] with lesser amounts of lauric acid (14:0), linoleic acid [18:2(n–6)] and others (Table 8). The sterols consisted almost entirely of cholesterol which is an essential constituent of crustacean diets. Chlorophyll *a* ranged from 0.6% (*N. oculata* CS–216) to 1.7% (*N. oculata* CS–179 and *N. salina*) of cell dry weight. Chlorophylls *b* and *c* were not detected. All strains contained a distinctive pattern of carotenoid pigments which included violaxanthin, β–carotene, zeaxanthin and a pigment tentatively identified as vaucheriaxanthin–ester. The distinctive pigment and lipid compositional data can be used as chemotaxonomic markers for *Nannochloropsis* and for assigning microalgae to the class Eustigmatophyceae. Based on the similarity of their biochemical data, both *N. salina* and the un–named tropical species should also prove to be nutritionally–valuable live algal feedstocks. Feeding trials will be needed to confirm this.

The high abundance of polar lipids, and low proportion of triacylglycerols, found in the two strains of *N. oculata* and in strain CS–246 is typical of microalgae grown under nutrient–sufficient conditions (e.g. Volkman *et al.*, 1989). High concentrations of triacylglycerols are commonly found in species grown to stationary phase when the nitrogen (nitrate) content of the medium is depleted (Hodgson *et al.*, 1991 and references therein). The higher abundance of triacylglycerols in *N. salina* is thus unusual since the cells were still actively growing when harvested.

The major fatty acids in the eustigmatophytes were 14:0, 16:0, 16:1(n–7) and 20:5(n–3). Similar fatty acid distributions have been reported previously for *N. oculata* (Maruyama *et al.*, 1986, Hodgson *et al.*, 1991), *N. salina* (Emdadi and Berland, 1989) and some unidentified *Nannochloropsis* strains (Suen *et al.*, 1987, Sukenik *et al.*, 1989). A similar pattern of abundance is also found in most diatoms, except that most diatoms contain some 22:6(n–3), which was absent from the eustigmatophytes, and a much higher content of C_{16} polyunsaturated fatty acids. Both algal groups lack significant amounts of C_{18} polyunsaturated fatty acids whereas in green algae these fatty acids are commonly major constituents. The close similarity between fatty acid biosynthetic pathways in diatoms and eustigmatophytes is further evident in the positions of the double bonds. In both classes of algae the 16:2 and 16:3 isomers have terminal double bonds at positions n–4 and n–7, whereas in green algae these double bonds occur at positions n–3 and n–6 (e.g. Volkman *et al.*, 1989, Dunstan *et al.*, 1992). Thus, despite superficial physical similarities between eustigmatophytes and some coccoid green algae, the two algal classes can be readily distinguished from their fatty acid compositions as well as from their pigment profiles.

Hodgson *et al.* (1991) showed that the abundance of 20:5(n–3) in *N. oculata* declined as the culture age increased, such that its relative proportion in stationary phase was only half that during exponential growth (15.4% cf 26.4–28.2%). This was accompanied by a build up of triacylglycerols which the authors presumed were deficient in 20:5(n–3), although paradoxically their data show that the content of 20:5(n–3) in triacylglycerols during exponential growth was actually quite high (34.9–37.5%, Hodgson *et al.*, 1991). In a similar way, the lower abundance of 20:5(n–3) in *N. salina* (Table 8) may reflect the much higher content of triacylglycerols present compared with the other eustigmatophytes, but triacylglycerols were not isolated to enable us to test this. As a general rule, while the compositional differences between lipid classes can be considerable, the fatty acid composition of an individual lipid classes tends to be more stable (particularly for structural lipids such as phospholipids) and hence of more value as a biochemical marker.

Our previous studies of the neutral lipids in these same four algal samples showed that the major sterol was cholesterol (Volkman *et al.*, 1992a). These algae lack C_{28} sterols and contain both E and Z isomers of the 24(28) double bond in C_{29} $\Delta^{5,24(28)}$–diunsaturated sterols which is quite unusual (Volkman, 1986). Suen *et al.* (1987) also identified cholesterol as the only major sterol present in another species of *Nannochloropsis* (strain QII). The presence of cholesterol, which is an essential constituent of prawn diets

(Teshima *et al.* 1983), rather than the complex suite of C–24 alkylated phytosterols common to most microalgae (Volkman, 1986), may be an advantageous feature for larval crustacean feeding. Also, all of these species contain high contents of vitamin C (Brown and Miller 1992).

Nannochloropsis salina is halotolerant and grows well in outdoor ponds. It has been extensively investigated as a source of biomass and lipids with growth rates up to 24.5 $g.m^{-2}.day^{-1}$ (Boussiba *et al.*, 1987). Lipid contents up to 74% of ash–free dry weight (as *Monallantus salina*, Shifrin and Chisholm 1981) have been achieved. *Nannochloropsis oculata* is widely used as a mariculture food species, and it also grows well under conditions of mass culture. Its small size, as well as its high concentrations of the essential polyunsaturated fatty acid 20:5(n–3), has made this alga particularly suited for feeding to the rotifer *Brachionus plicatilus*, the brine shrimp *Artemia salina* (for fish larviculture: Watanabe *et al.* 1983, Sato 1991), and as food for larvae of the oyster *Crassostrea virginica* Gmelin (Dupuy 1975). The complete absence of C_{22} polyunsaturated fatty acids does not appear to limit the nutritional value of this species. This alga is a possible source of oils rich in the 20:5(n–3) fatty acid for use as a food supplement (e.g. Borowitzka and Borowitzka, 1988, Gladue, 1991). The lack of 22:6(n–3) would be an advantage in studies designed to investigate the requirement for specific polyunsaturated fatty acids in the diet of marine animals.

In our study we compared the biochemical composition of the tropical eustigmatophytes CS–246 and *N. salina* with those of temperate *N. oculata* which has proven to be so successful as a mariculture feedstock. In view of the small differences observed in the gross biochemical compositions, it seems likely that these two eustigmatophytes could also be used successfully as live microalgal feeds particularly in tropical environments. The slightly higher content of carbohydrate in *N. salina* may be of nutritional significance for the feeding of molluscs (Whyte *et al.*, 1989). Although *N. salina* contains proportionally only half as much 20:5(n–3) fatty acid as the other species, the amount per cell is actually higher and would clearly meet the dietary requirements of most marine animals.

Differences in the sugar composition of polysaccharides from microalgae may be of nutritional significance, since the efficiency with which marine animals digest polysaccharide is dependent on the type present (Kristensen 1972, Onishi *et al.* 1985). The glucose–rich polysaccharides found in eustigmatophytes should be effectively broken down by the digestive enzymes of molluscs and crustaceans (Kristensen 1972). The total carbohydrate concentrations are not high, but Thomas *et al.* (1984) demonstrated that the carbohydrate yield in nitrogen–deficient cultures of *N. salina* (as *Monallantus salina*) can be doubled at the expense of protein synthesis with no change in lipid content. However, this result differs from the findings of Shifrin and Chisholm (1981) and needs to be tested by further study.

Algal protein is considered to be of high nutritional value if its essential amino acid composition is similar to that of the feeding animal (Webb and Chu, 1983). The amino

acids essential for fish and crustaceans include valine, leucine, isoleucine, lysine, histidine, arginine, tryptophan, phenylalanine, threonine and methionine (Harrison, 1975; Kanazawa and Teshima, 1981; Cowey and Tacon 1983), while molluscs also require proline (Harrison, 1975). The concentrations of the essential amino acids in the eustigmatophytes were, in most instances, either equivalent to or greater than the levels in larvae of the oyster *Crassostrea gigas* Thunberg (Volkman *et al.*, 1993) suggesting that they should provide a high quality protein for larval molluscs. The lysine concentrations are low, but this is also true of many other species of marine microalgae that have been used successfully as feedstocks (Brown, 1991; Brown and Jeffrey, 1992).

The fatty acids distributions in all strains were also very similar; the major fatty acids being 16:0, 16:1(n–7) and 20:5(n–3) (unpublished data). Similar fatty acid distributions have been reported previously for *N. oculata* (Maruyama *et al.*, 1986) and some unidentified strains (Sukenik *et al.*, 1989; Suen *et al.*, 1987). Very long–chain unsaturated ketones, as found in some species of prymnesiophytes (Marlowe *et al.*, 1984), were not present. The concentrations of hydrocarbons were too low to permit detailed studies. In contrast, Emdadi and Berland (1989) reported that hydrocarbons comprised 78% of total lipids in *N. salina* when harvested after just one days growth, but dropped to 2.3% after 16 days.

Our studies also showed that these microalgae contain unusual C_{30} – C_{32} 1,15–alkyl diols and a monounsaturated C_{32} 1,15–alkyl diol (Volkman *et al.*, 1992a). Alkyl diols are abundant in many marine sediments, but this is the first report of their occurrence in cultured microalgae. The diols do not occur as free lipids in the eustigmatophytes, at least under the culture conditions used, but they could be liberated from more polar lipids by acid hydrolysis. Acid hydrolysis also liberated C_{30} – C_{32} mono– and diunsaturated straight–chain alcohols. The carbon number distributions of the alcohols and diols are identical, and the relative proportions of homologues are similar, suggesting that both compound classes are formed by the same biosynthetic pathway. The nutritional significance of these compounds is presently unknown, but it appears that marine animals are able to utilise them when present in their diet.

In summary, the neutral lipid, fatty acid and pigment data show that the eustigmatophytes have a distinctive and characteristic biochemistry which we suggest might prove to be a good chemotaxonomic marker for this class of microalgae. Further study is required to establish the taxonomic affinities of the un–named eustigmatophyte (CS–246) from Queensland waters, but based on its lipid composition and cell morphology it appears to be closely related to *N. oculata*. These biochemical data confirm the high value of eustigmatophytes as live algal feeds for mariculture, but feeding trials with appropriate target animal species are still needed.

Table 7. Contents of total carbohydrates, proteins, lipids and chlorophyll *a* in four cultures of eustigmatophytes from the genus *Nannochloropsis*.

Species	*N. salina*	*N. oculata*	*N. oculata*	Un-named
CSIRO strain number	CS-190	CS-216	CS-179	sp. CS-246
Culture medium	fE	fE	f/2	f/2
Cells ml^{-1} at harvest (x 10^6)	5.3	9.5	14.7	8.0
Dry weight ($pg.cell^{-1}$)	8.3	9.3	3.1	5.3
Weight of constituent ($pg.cell^{-1}$)				
Total carbohydrate	0.73	0.60	0.16	0.31
Mono- and oligo-saccharides	0.13	0.083	0.027	0.04
Polysaccharide	0.60	0.52	0.13	0.27
Total protein	1.5	2.1	0.62	1.1
Total lipids	1.4	0.76	0.34	0.70
Polar lipids	0.76	0.66	0.32	0.65
Triacylglycerols	0.60	0.06	0.006	0.005
Sterols	0.04	0.04	0.019	0.026
Chlorophyll *a*	0.14	0.056	0.051	0.077

The average coefficients of variation based on previous work (Brown *et al.*, 1991) were as follows: cells ml^{-1} – ±9%; dry wt. – ±2.5%, carbohydrate pg $cell^{-1}$ – ±9.6%; protein pg $cell^{-1}$ – ±9.6%; lipid pg $cell^{-1}$ – ±9.9%; chlorophyll *a* pg $cell^{-1}$ – ±9.2%. These calculations take into account the error in cell counts.

Table 8. Percentage compositions and concentrations of total fatty acids in eustigmatophytes

Species	*N. salina*	*N. oculata*	*N. oculata*	Un-named sp.		
Code	CS-190	CS-216	CS-179	CS-246	CS-246	CS-246
Temperature ^{o}C	20	20	20	27	27	27
Extraction method*	A	A	A	A[a]	A[a]	B
Saturated fatty acids						
14:0	5.0	3.3	4.6	5.4	5.1	5.5
15:0	0.5	0.4	0.5	0.6	0.3	0.4
16:0	27.8	17.8	14.2	20.1	21.6	27.0
18:0	1.0	0.9	0.6	0.6	0.5	0.8
Subtotal	34.5	22.4	20.0	26.8	27.5	33.7
Monounsaturated fatty acids						
16:1(n-9)	0.1	–[b]	0.1	tr	–	–
16:1(n-7)	31.8	26.6	29.4	20.9	22.1	23.3
16:1(n-5)	0.4	0.2	0.2	0.1	0.1	0.1
16:1(n-13)*t*	0.1	0.3	0.4	0.5	0.8	0.9
17:1(n-8)	0.2	0.7	0.8	0.3	0.1	0.1
18:1(n-9)	8.3	7.7	6.3	4.6	2.2	2.3
18:1(n-7)	0.2	0.9	0.3	0.5	0.6	0.7
Subtotal	41.1	36.4	37.4	26.9	25.9	27.5
Polyunsaturated fatty acids						
16:2(n-7)	0.3	0.4	0.8	0.7	0.8	0.6
16:2(n-4)	0.1	0.1	0.1	0.2	0.4	0.4
16:3(n-4)	0.1	0.1	0.2	tr	0.2	0.1
18:2(n-9)	0.2	0.2	0.3	0.2	tr	tr
18:2(n-6)	1.5	2.9	2.0	2.7	2.3	2.2
18:3(n-6)	0.4	0.3	0.3	0.8	0.9	0.8
18:3(n-3)	0.2	0.4	0.1	0.9	0.1	0.1
20:3(n-6)	0.9	0.2	0.4	0.1	0.3	0.3
20:3(n-3)	tr	0.1	0.1	0.2	0.3	0.3
20:4(n-6)	4.0	7.1	8.8	6.4	5.9	5.1
20:5(n-3)	16.1	28.4	28.8	33.1	34.4	28.0
22:6(n-3)	–	–	–	–	–	–
Subtotal	24.2	40.8	42.2	45.7	46.0	38.2
Others	0.5	0.9	0.8	0.9	0.9	0.9
pg FA cell^{-1}	0.93	0.53	0.27	0.39	0.44	0.34
mg FA g^{-1} dry wt.	112	82	88	–	83	64

A: Bligh and Dyer extraction; B: Bligh and Dyer extraction with addition of acid to the solvents. Subtotals include contributions from minor fatty acids not listed. The averages of the co-coefficients of variation were ±2.0% for mean values >1.0% and ±3.5% for mean values <1.0%).

[a] replicate cultures grown 3 months apart

[b] – indicates that the fatty acid was not detected (<0.05% of total fatty acids)

5.1.4 Lipids of prymnesiophytes

Summary

Prymnesiophytes of the genus *Pavlova* and *Isochrysis* have been widely used as mariculture feeds and our studies confirmed that they are excellent sources of PUFA (Volkman *et al.*, 1992b; Brown *et al.*, 1993). However, the class contains a great diversity of lipid compositions and many species contain unusual lipids such as steroidal diols or long-chain ketones. *Pavlova* species in particular contain high contents of both 20:5(n-3) and 22:6(n-3) PUFA. The temperate species *Pavlova lutheri* is widely used in Australia, but our studies have identified alternative species better suited for tropical conditions. *Isochrysis* on the other hand contains 22:6(n-3), but only small amounts of 20:5(n-3). The strain from Tahiti (called T.iso) has largely replaced the species *I. galbana* for use in Australia.

Microalgae from the class Prymnesiophyceae (Haptophyceae) are common in the marine environment and important contributors of organic matter to marine food webs. The lipids of Prymnesiophyceae have been intensively studied following the recognition that a few species synthesize large amounts of unusual very long-chain unsaturated ketones and alkenes (Volkman *et al.*, 1980; Marlowe *et al.*, 1984). The class Prymnesiophyceae contains the orders Isochrysidales, Coccosphaerales, Prymnesiales and the Pavlovales.

Species from the genera *Isochrysis* and *Pavlova* (class Prymnesiophyceae) are particularly popular for use as larval feeds (De Pauw, 1981). Within Australia, the Tahitian strain of *Isochrysis* (commonly referred to as T.iso) is widely used in mariculture. This strain has largely replaced *Isochrysis galbana* world-wide as a food for bivalve molluscs (Helm and Laing, 1987). *Isochrysis* sp. (T.iso) contains significant proportions of PUFA, such as 22:6(n-3), and has often been used successfully as a feedstock for marine animals (e.g. Helm and Laing, 1987). Although 20:5(n-3) is not abundant in many prymnesiophytes it is found in significant amounts in another the genus *Pavlova* (Volkman *et al.*, 1991).

The major fatty acids of *Isochrysis* sp. (T.iso) were 18:4(n-3), 14:0, 18:1(n-9), 16:0 and 22:6(n-3) (Enright *et al.* 1986a; Pillsbury 1985; Volkman *et al.*, 1989). A distinctive feature of these fatty acid compositions is the very low abundance of 20:5(n-3) and high abundance of 22:6(n-3). There have been at least 7 detailed fatty acid analyses of *Isochrysis galbana*, but there is little agreement concerning the relative abundances of major fatty acids (reviewed by Volkman, 1989). The percentage of 20:5(n-3) and 22:6(n-3) reported in *I. galbana* ranged from 0 to 14.4% and 0 to 18.9% of the total fatty acids respectively. Such differences may account, in part, for the diversity of views concerning the value of *I. galbana* as a mariculture food (Helm and Laing, 1987).

An unusual feature of the lipid composition of *Isochrysis* spp. and a few other prymnesiophytes such as *Emiliania huxleyi* is the presence of very long chain C_{37}–C_{39} unsaturated ketones, hydrocarbons and methyl and ethyl esters of a 36:2 fatty acid (Volkman *et al.*, 1980; Marlowe *et al.*, 1984). These compounds are not assimilated by the copepod *Calanus helgolandicus* when fed *Emiliania huxleyi* (Volkman *et al.*, 1980), although we have found large amounts in *Artemia* fed on *Isochrysis* (unpublished results). High concentrations of these waxy compounds in the animal's diet may act as a laxative, and it is likely that they are poorly metabolized by most marine animals. This feature does seem to detract significantly from their value as a live feed for aquaculture and it has been suggested that they may be useful as antioxidants in mariculture feeds and reduce feed spoilage (Ben Amotz *et al.*, 1991).

Another widely used prymnesiophyte is *Pavlova lutheri* (Nichols *et al.*, 1989). Species of *Pavlova* are typically small (5 – 8 μm) flagellates which are widely distributed in oceanic, coastal and brackish waters. Several species of *Pavlova* are common in Australian waters. Only one species of *Pavlova* is known from a fresh water lake. Members of this genus lack the calcium carbonate coccoliths and unmineralized scales found in many other species of the Prymnesiophyceae. The species of *Pavlova* analysed here belong to the Pavlovales, the taxonomy of which has been subject to many changes over recent years. *Pavlova lutheri* was originally named *Monochrysis lutheri* (Droop) and included within the Chrysophyceae, but later studies using electron microscopy showed the presence of a haptonema and flagellar organization characteristic of the Prymnesiophyceae. These and other structural features as well as characteristic pigment compositions are now routinely used to assign species to the Prymnesiophyceae or Chrysophyceae.

Pavlova lutheri is widely used as an algal food for larval animals in mariculture hatcheries. It grows well in culture and is widely used as food for larval animals in the mariculture industry in cool–temperate to subtropical climates (10 – 25 ^{o}C). *P. lutheri* is unsuitable for use in tropical hatcheries, but could be replaced by other species such as *P. salina* which grows well at 30 ^{o}C. In order to test this, the lipid and fatty acid composition of four species of *Pavlova* were determined.

Total lipid content in each species were very similar in the range 4.5 to 8.0 pg per cell (Table 9). Triacylglycerol contents were generally low with polar lipids dominating the lipid profiles. The major fatty acids (Table 10) were 14:0, 16:0, 16:1(n–7), 20:5(n–3), 18:4(n–3) and 22:6(n–3) which comprised 83.6–89.8 % of the total fatty acids in each species. These distributions are similar to those found in diatoms, except that *Pavlova* species contain much higher abundances of 18:4(n–3) and 22:6(n–3). The distinctive fatty acid, sterol and pigment compositions of *Pavlova* species sets them apart from other groups within this class. From these and other data, it appears that the Prymnesiophyceae contains a greater biochemical diversity than is found in many other classes of microalgae.

Species of *Pavlova* did not contain long–chain unsaturated ketones, but they do synthesize unusual 4–methyl sterols and steroidal diols (Volkman *et al.*, 1990). Their major fatty acids are 16:0, 16:1(n–7), 20:5(n–3) and 22:6 (n–3) which is a very different distribution from that of *Isochrysis* sp. (T.iso), and more closely resembles that of diatoms except for the higher abundance of 18:4(n–3) and 22:6(n–3) (Chuecas and Riley, 1969; Chu and Dupuy, 1980; Langdon and Waldock, 1981; Volkman *et al.*, 1991). The high abundances of C_{20} and C_{22} PUFA make these species particularly useful foods. Rotifers fed on *P. lutheri* accumulate high levels of 20:5(n–3), 22:6(n–3) and other PUFA within a few hours (Nichols *et al.*, 1989), which provides a convenient means of transferring these acids to fish larvae.

P. salina contains similar amounts of polyunsaturated fatty acids to *P. lutheri* when grown at 20 ^{o}C, and there was only a small diminution of PUFA content in *P. salina* when grown at 27 ^{o}C (Table 10). The other isolates of *Pavlova* also grow well at 30 ^{o}C and could substitute for *P. lutheri* as a mariculture food species provided that they do not contain toxins. *Pavlova salina* and the two tropical isolates of *Pavlova* sp. have a similar fatty acid profile and even higher levels of 20:5(n–3) and 22:6(n–3) (Volkman *et al.*, 1991). These species grow well at 30 ^{o}C and thus will be useful replacements for *P. lutheri* in tropical hatcheries.

Other species within this algal class display a diversity of distributions with 16:0, 16:1(n–7), 18:1(n–9), 18:4(n–3), 20:5(n–3) and 22:6(n–3) fatty acids being most common (Holz, 1981; Marlowe *et al.*, 1984; Volkman *et al.*, 1981, 1989a,b). Such diversity of biochemical compositions is unusual and probably reflects the heterogeneity of algal types that are included in this taxonomic grouping (Volkman *et al.*, 1990). We also carried out further studies of the lipids in prymnesiophytes with the assistance of a University of Tasmania B.Sc.(Hons) student Ms Christine Farmer. A local isolate of *Emiliania huxleyi* was examined and significant differences in the proportions of key lipids were found compared with the previously studied isolate from the Sargasso Sea.

Table 9. Total lipid content and percentage composition of lipid classes in prymnesiophytes determined by Iatroscan thin layer chromatography–flame ionization detection.

Species	Strain	Total lipids (pg/cell)	Percentage Concentrations						
			HC/WE	TG	FFA	4Me	ST	SD	PL
P. salina	CS–49	4.5	0.4	1.2	0.8	3.7	3.1	2.2	88.6
Pavlova sp.	CS–50	5.8	tr	6.6	tr	0.7	4.5	0.8	87.3
Pavlova sp.	CS–63	5.7	tr	10.7	tr	0.5	4.2	0.6	84.0
P. lutheri	CS–182	8.0	0.5	5.8	tr	2.1	6.3	12.5	72.8
*P. lutheri***	CS–182	nd	0.2	4.0	tr	*	6.3	11.0*	78.3

HC/WE = hydrocarbons plus wax esters; TG = triacylglycerols; FFA = free fatty acids; 4Me = 4–methyl sterols; ST = sterols; SD = steroidal diols?; PL = polar lipids and chlorophylls; tr = less than 0.2%; * = SD includes 4–methyl sterols; ** = results from Volkman *et al.* (1989a).

Table 10. Relative concentrations of total fatty acids in species of *Pavlova*.

Species	*P. salina*	*P. salina*	*P. salina*	*Pavlova* sp.	*Pavlova* sp.	*Pavlova* sp.	*Pavlova* sp.	*P. lutheri*	*P. lutheri*
Strain	CS-49	CS-49	CS-49	CS-50	CS-50	CS-63	CS-63	CS-182	CS-182
Temp (°C)	27	20	20	20	20	20	20	20	20
Sample	A	A	B	A	B	A	B	B	*
Saturated fatty acids									
14:0	17.8	16.0	14.0	16.5	19.0	22.1	22.8	13.4	11.9
15:0	0.5	0.3	0.3	0.4	0.3	0.3	0.2	0.4	0.5
16:0	18.5	14.1	16.3	13.1	12.5	11.3	11.9	17.7	22.0
18:0	0.2	0.1	0.2	0.6	0.3	0.3	0.3	0.7	1.3
subtotal	37.1	30.5	30.8	30.6	32.1	34.1	35.2	32.2	35.8
Monounsaturated fatty acids									
16:1(n-7)	4.3	5.4	5.2	10.8	9.2	10.1	10.2	14.4	17.4
16:1(n-5)	0.1	0.1	0.1	2.5	2.7	3.5	3.3	–	tr
16:1(n-13)*t*	nd	nd	0.1	nd	0.4	nd	tr	0.2	–
18:1(n-9)	0.7	0.2	0.4	0.7	0.4	0.5	0.4	1.6	1.8
18:1(n-7)	tr	tr	–	0.2	0.3	0.1	0.2	1.2	1.4
20:1	tr	tr	tr	tr	tr	tr	0.1	tr	0.2
subtotal	5.2	5.8	5.8	14.2	13.0	14.2	14.1	17.4	20.8
Polyunsaturated fatty acids									
16:2(n-7)	0.2	0.1	–	0.1	–	0.1	–	0.3	0.2
16:2(n-4)	0.3	0.3	0.8	0.1	1.7	0.1	1.2	0.5	0.2
16:3(n-4)	0.2	0.2	0.1	0.4	0.3	0.3	0.2	0.3	0.4
18:2(n-9)	tr	tr	tr	tr	tr	–	tr	0.3	0.4
18:2(n-6)	2.0	1.8	1.9	1.1	1.0	1.8	1.5	2.3	1.6
18:3(n-6)	2.4	0.7	1.2	0.5	0.4	0.5	0.6	1.1	0.4
18:3(n-3)	1.1	1.3	1.4	2.0	1.4	1.4	1.1	1.6	1.9
18:4(n-3)	10.7	15.2	13.6	11.2	11.0	9.3	9.5	7.5	6.2
20:4(n-6)	0.7	0.4	0.8	0.6	0.7	0.6	0.6	0.6	tr
20:5(n-3)	25.4	28.2	25.8	25.0	23.5	24.2	21.5	22.4	20.4
22:5(n-6)	3.8	4.5	7.4	4.8	6.4	4.2	6.9	2.7	2.1
22:6(n-3)	11.0	10.9	10.2	9.2	8.4	9.3	7.7	10.7	9.7
subtotal	57.8	63.7	63.3	55.2	54.9	51.7	50.7	50.4	43.4
Concentration (pg/cell)	nd	nd	2.7	nd	2.6	nd	2.6	3.0	3.2

nd, not determined; tr, trace; *, values recalculated from data reported by Volkman *et al.* (1989)

5.1.5 Lipids of Cryptomonads and a Rhodophyte

Summary

Fatty acid, sterol and lipid class analyses of seven strains (eight samples) of the little studied microalgal class Cryptophyceae were completed to identify potential new species with nutritional and growth characteristics required for the mariculture industry. All strains, including two new Australian isolates which grow well in culture, contain significant levels of both 20:5(n–3) and 22:6(n–3) PUFA and some have favourable growth characteristics for either temperate and tropical hatchery use. A manuscript describing these results has been prepared for publication (Dunstan *et al.*, 1995).

Cryptomonads are not widely used as mariculture feeds, except for *Chroomonas salina* which is used in Australia mainly for the rearing of oysters. Its major fatty acids are 18:4(n–3), 16:0, 18:3(n–3), 18:2(n–6) and 20:5(n–3) (Volkman *et al.*, 1989). The high proportion of C_{18} PUFA, 20:5(n–3) and total (n–3) PUFA (36–77%; Beach *et al.*, 1970), together with the very low abundance of C_{16} PUFA is typical of species from this class (Holz, 1981; Beach *et al.*, 1970; Chuecas and Riley, 1969). Two species are reported to lack 20:5(n–3) and 22:6(n–3) (reviewed by Beach *et al.*, 1970). *Rhodomonas* sp. was found to be a good food for juvenile oysters, which Enright *et al.* (1986) thought might be due to its high carbohydrate content.

Species from the Rhodophyceae have only infrequently been used as foods in mariculture. Species from this class have been of considerable interest to lipid biochemists because of their very high concentrations of arachidonic acid [20:4(n–6)], which is a precursor to prostaglandins such as PGE_2. Other major acids are 16:0, 18:2(n–6) and 20:5(n–3) (Nichols and Appleby, 1969). In *Porphyridium cruentum*, 20:4(n–6) and 20:5(n–3) represented 36 and 17% of the total fatty acids, 26% and 40% of the fatty acids in monogalactosyl diglycerides and 59% and 10% in phosphatidyl choline (Nichols and Appleby, 1969). These proportions can be varied by changing culture conditions so that 20:5(n–3) becomes the major fatty acid (Cohen *et al.*, 1988).

Based on literature data, cryptophytes would appear to be good candidates as algal feedstocks. In order to test this, seven strains of marine cryptomonad harvested at logarithmic phase were analysed to determine their lipid class, fatty acid and sterol compositions. The major lipid classes in all strains harvested at this growth phase were the polar lipids (Table 11). Most strains contained high proportions of the (n–3) polyunsaturated fatty acids, 18:3(n–3) (20.7–29.9%), 18:4(n–3) (12.5–30.2%), 20:5(n–3) (7.6–13.2% of total fatty acids) and 22:6(n–3) (6.4–10.8%) (Table 12). The blue-green cryptomonad *Chroomonas placoidea* was unusual because it contains a very small amount

of 22:6(n–3) (0.2% of total fatty acids), whereas 22:5(n–6) was a significant component (4.5%). For comparison, one strain of a microalgal rhodophyte, *Rhodosorus* sp. was examined. The fatty acid composition of the rhodophyte was similar to those of the Cryptophyceae except for decreased levels of 18:4(n–3) and no C_{22} PUFA were not detected (Table 12).

The major sterol in the Cryptophyceae and the Rhodophyceae was 24–methylcholesta–5,22E–dien–3β–ol (62.4–99%); this sterol is also abundant in many diatoms. Small amounts of cholesterol (1–17.7 %) were present in all the cryptophytes (Table 13), but it was not present in *Rhodosorus*. *Chroomonas placoidea* contained an unusually high content of the C_{29} sterol 24–ethylcholesta–5,22E–dien–3β–ol.

Table 11: Species of Cryptophyceae and Rhodophyceae, their phycobilin type, growth temperatures, cell count at harvest, and percentage composition of lipid classes

Species	CSIRO Culture Code	Phycobilin type	Growth temperature (^{o}C)	cell counts X10^5	Lipid classes (Percentage Composition)[a] PL	PG	ST	FFA	TG	HC/WE
CRYPTOPHYCEAE										
Chroomonas placoidea	CS–200	phycocyanin	15	3.53	78	1.4	1.7	3.6	13	2.1
Chroomonas salina	CS–174	phycoerythrin	20	3.55	85	2.8	2.3	7.0	–[b]	2.6
Chroomonas salina	CS–174	phycoerythrin	20	3.73	84	3.2	2.2	7.3	–	3.6
Chroomonas sp.	CS–24	phycoerythrin	20	3.58	85	3.5	1.2	9.2	–	1.5
Cryptomonas maculata	CS–85	phycoerythrin	20	2.65	86	5.1	1.7	5.4	–	2.2
Rhodomonas sp.	CS–215	phycoerythrin	20	3.05	88	2.7	2.3	5.0	–	1.7
Cryptomonad PCL	*	phycoerythrin	15	5.05	87	1.5	4.6	3.7	–	2.9
Cryptomonad QLD	*	phycoerythrin	25	12.5	86	2.6	3.6	4.7	–	3.0
RHODOPHYCEAE										
Rhodosorus sp.	CS–210	phycoerythrin	25	nd[c]	nd	nd	nd	nd	nd	nd

[a] PL, polar lipids and chlorophylls; PG, pigments, ST, sterols; FFA, free fatty acids; TG, triacylglycerols; HC/WE, hydrocarbons/wax esters.
[b] not detected; [c] no data because samples were saponified.

Table 12. Relative concentrations of fatty acids and total cellular fatty acid content in seven strains of microalgae from the Cryptophyceae and one from the Rhodophyceae

Species	*Chroomonas placoidea*	*Chroomonas salina*	*Chroomonas salina*	*Chroomonas* sp.	*Cryptomonas maculata*	*Rhodomonas* sp.	PCL Pipeclay lag.	25 C Qld.	*Rhodosorus* sp.
Code	CS-200	CS-174(1)	CS-174(2)	CS-24	CS-85	CS-215	PCL *	Qld	CS-210
Saturated fatty acids									
14:0	1.0	7.3	7.4	3.8	7.3	6.7	2.1	3.1	0.3
15:0	0.1	0.2	0.2	0.1	0.3	0.2	0.2	0.2	0.1
16:0	16.2	8.3	8.0	8.6	10.1	8.2	11.4	11.6	26.9
18:0	1.4	0.6	0.5	1.0	0.7	0.7	1.2	0.4	1.1
Subtotal	18.7	16.4	16.1	13.5	18.4	15.8	14.9	15.2	28.3
Monounsaturated fatty acids									
16:1(n-9)	0.4	0.5	0.4	0.2	0.3	0.5	0.7	0.7	0.1
16:1(n-13)t	1.8	1.9	2.0	2.1	2.0	2.4	2.1	1.7	0.9
16:1(n-7)	1.8	1.0	1.0	0.5	0.7	0.9	0.9	1.4	1.2
16:1(n-5)	0.1	tr	tr	0.1	0.1	tr	0.1	0.1	0.2
18:1(n-9)	2.8	0.7	0.6	0.5	0.9	0.6	0.9	1.0	9.3
18:1(n-7)	6.7	2.6	2.7	6.5	4.4	6.0	1.7	1.3	0.6
18:1(n-5)	–	tr	tr	0.4	tr	0.5	0.4	tr	–
20:1(n-9)	0.1	–	–	tr	–	–	0.1	tr	0.1
Subtotal	13.6	6.8	6.7	10.3	8.4	10.8	6.8	6.2	12.4
Polyunsaturated fatty acids									
16:2(n-4)	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.1
18:2(n-6)	9.3	14.7	14.0	1.4	2.1	5.3	0.5	2.9	5.7
18:3(n-6)	0.4	2.3	2.4	0.3	0.2	0.6	0.2	0.5	0.5
18:3(n-3)	27.2	21.3	21.6	20.7	26.6	29.9	22.0	29.3	26.5
18:4(n-3)	12.5	18.4	19.2	30.2	27.5	20.9	30.0	20.6	0.8
18:5(n-3)	0.4	–	–	–	–	0.1	–	–	–
20:2(n-6)	0.1	0.1	0.1	tr	0.1	tr	0.1	tr	0.1
20:3(n-6)	tr	0.1	0.1	–	–	–	tr	tr	0.1
20:4(n-6)	0.3	2.4	2.6	0.2	0.2	0.6	0.2	0.6	3.7
20:3(n-3)	0.1	tr	tr	0.1	0.1	tr	0.1	0.1	0.5
20:4(n-3)	0.4	0.5	0.5	0.5	0.3	0.2	0.4	0.4	0.4
20:5(n-3)	11.0	9.3	9.1	13.2	7.7	7.6	12.3	12.6	20.7
21:5(n-3)	0.3	0.2	0.2	tr	tr	0.2	0.4	0.1	–
22:5(n-6)	4.5	0.3	0.3	0.1	0.4	0.1	0.6	2.4	–
22:5(n-3)	–	0.1	0.1	0.2	0.1	0.1	0.2	0.2	–
22:6(n-3)	0.2	6.5	6.4	7.8	6.4	6.7	10.8	7.8	–
Subtotal	66.8	76.1	76.6	74.9	71.9	72.3	77.8	77.6	58.9
Others[a]	0.9	0.6	0.6	1.3	1.3	1.1	0.4	0.9	0.3
Total FA pg/cell	4.7	6.5	6.0	7.4	11	8.7	5.1	2.2	nd

Table 13. Relative concentrations of sterols and total cellular sterol content in seven strains of microalgae from the Cryptophyceae and one from the Rhodophyceae

Species	*Chroomonas placoidea*	*Chroomonas salina*	*Chroomonas salina*	*Chroomonas* sp.	*Cryptomonas maculata*	*Rhodomonas* sp.	PCL Pipeclay lag.	25 C Qld.	*Rhodosorus* sp.
Code	CS-200	CS-174(1)	CS-174(2)	CS-24	CS-85	CS-215	PCL *	Qld	CS-210
Sterol (% of total sterols)									
cholest-5-en-3β-ol	2.0	1.1	1.0	1.9	17.7	1.3	1.0	2.7	–
24-methylcholesta-5,22E-dien-3β-ol	62.5	98.9	99.0	98.1	82.3	98.7	91.5	97.3	71.8
24-methylcholesta-5,7,22-trien-3β-ol	–	–	–	–	–	–	7.5	–	–
24-methyl-5α-cholesta-7,22E-dien-3β-ol	–	–	–	–	–	–	–		2.2
24-methylcholest-5-en-3β-ol	–		–	–	–	–	–	2.5	–
4-methyl-5α-cholesta-7,22-dien-3β-ol	–	–	–	–	–	–	–	–	12.3
24-ethylcholesta-5,22E-dien-3β-ol	35.5	–	–	–	–	–	–	–	–
24-methyl-5α-cholest-7-en-3β-ol	–	–	–	–	–	–	–	–	3.4
4,24-dimethyl-5α-cholest-7-en-3β-ol	–	–	–	–	–	–	–	–	5.8
others								2.0	–
sterols pg/cell	0.14	0.20	0.20	0.14	0.23	0.16	0.13	0.05	nd

*: isolated from phytoplankton at Pipe Clay Lagoon, Tasmania.

5.2 Studies of the effects of different culture conditions on biochemical composition (Objective 3)

Summary

Previous studies have shown that the abundance of the various lipid classes and proportions of PUFA can be altered by changes in environmental conditions, but there have been few systematic or detailed investigations. We therefore undertook studies of how biochemical compositions are influenced by changes in light intensity, nutrient composition and stage of growth at harvest etc., in order to identify factors which maximize PUFA content.

Large scale (100 litre bag) culture of the microalgae *Pavlova lutheri*, *Nannochloropsis oculata* and *Isochrysis* sp. which are often used for mariculture and biotechnological purposes, were established. The microalgae were harvested semi-continuously (a common practice in hatcheries, where a proportion of the culture is removed for use and replaced with fresh media), or as a batch (where the whole culture is harvested in its entirety), and at both logarithmic and stationary phase to examine the effect on lipid composition and in particular the PUFA content. These results are discussed in Dunstan *et al.* (1993).

A total of 33 samples were analysed from twelve bag cultures. Maximum proportion and cellular content of 20:5(n-3) was found in *N. oculata* and *P. lutheri* when harvested as a batch culture, but by allowing the cultures of *P. lutheri* to reach stationary phase it was possible to significantly increase the amount of the essential fatty acid 22:6(n-3) present [note that *N. oculata* does not normally contain 22:6(n-3)]. Unexpectedly, in *Isochrysis* sp. (T.iso) there was no change in the cellular content of these fatty acids during either treatment. In all stationary phase samples the proportion of PUFA decreased with time, due primarily to the increase of lower PUFA storage lipid within the cells. These data indicate that hatcheries can optimize the nutritional quality of algal feedstocks, particularly the PUFA content, by modifying the way in which the microalgae are cultured and harvested.

Growth conditions can have a marked effect on the biochemical composition of microalgae, but the effects vary from one species to another and the information available is still too limited to make broad generalizations. Major changes in fatty acid composition

have been documented when nutrients are limited or when the temperature, light intensity or light/dark cycle are changed (Borowitzka and Borowitzka, 1988; Ballantine *et al.*, 1979; Mortensen *et al.*, 1988). It is a common observation that cells in stationary phase accumulate high lipid contents (particularly triacylglycerols) as nutrients become depleted (Ballantine *et al.*, 1979; Webb and Chu, 1983; Borowitzka and Borowitzka, 1988). Under nutrient–sufficient conditions, rapidly growing cells incorporate most carbon into protein, but when nitrogen is limiting most microalgae increase lipid biosynthesis.

The lipid content in diatoms increases as soon as silicate becomes a limiting nutrient (Enright *et al.*, 1986b; Taguchi *et al.*, 1987), although this can be accompanied by reduced levels of PUFA (Mortensen *et al.*, 1988). Lipid concentrations, and content of PUFA, increases dramatically in the dark in some species (Mortensen *et al.*, 1988; Sicko–Goad *et al.*, 1988). Temperature also has an effect on the relative proportions of unsaturated fatty acids, with an increase of unsaturation occurring at lower temperatures (Mortensen *et al.*, 1988). James *et al.* (1989) noted that the content of 20:5(n–3) in *Nannochloropsis* sp. increased from 19% at 15 ^{o}C to 23.7% at 25 ^{o}C, followed by a decline to only 7.4% at 35 ^{o}C. The content of 20:5(n–3) decreased, and that of 20:4(n–6) increased, when the growth rate of *Porphyridium cruentum* in culture was reduced by decreasing light intensity, increasing cell concentration or salinity or using sub–optimal temperatures or pH (Cohen *et al.*, 1988).

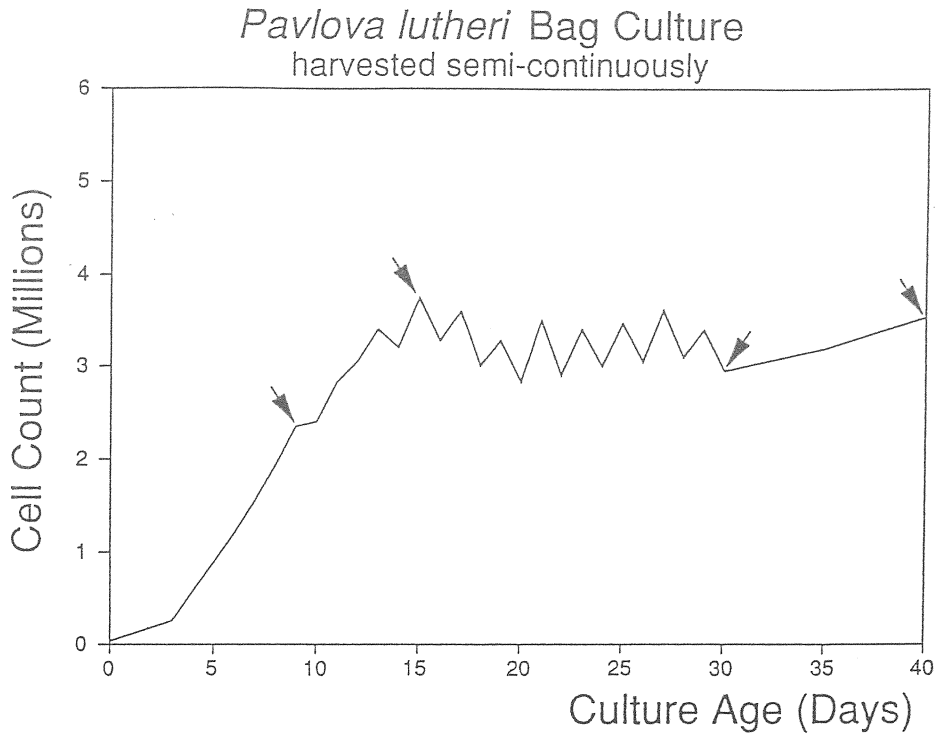
5.2.1 Biochemical changes with growth stage

Summary

Three species of microalgae were grown in mass culture to investigate the influence of culture technique and growth phase on the production of 20:5(n–3) and 22:6(n–3). The species of microalgae examined were *Nannochloropsis oculata*, *Pavlova lutheri* and *Isochrysis* sp. (T.Iso). All batch cultures (logarithmic and stationary phase) and semi–continuous cultures (logarithmic phase) examined contained high levels of the long–chain (n–3) PUFA, but production of these could be increased by harvesting at specific times and growth phases. The maximum cellular content (pg cell^{-1}) of long–chain PUFA was found in logarithmic phase batch cultures of *N. oculata* and in stationary phase cultures of *P. lutheri*.

Microalgae were cultured in two sets of duplicate 100 litre polyethylene bags under conditions which are similar to those commonly used in hatcheries. Each contained 85 litres of f/2 medium which were inoculated with 1.2 litres of axenic starter culture. They were aerated with food–grade air supplemented with CO_2 (0.5% by volume)at a rate of 20

ml $min.^{-1}$ and grown with a 12:12 light:dark photoperiod. The bags were set up under controlled temperature conditions at 22 °C at the research facility at Pipeclay Lagoon. Samples were taken at 10 am to 12 am to minimise any diurnal variations. One set of bags was harvested at 9 days and 23 days (Figure 8), while the other was harvested at 9 days and then harvested semicontinuously by removing 15% of the culture volume and adding fresh media to maintain an approximately constant cell count. Cell counts for *P. lutheri* are shown in Figure 8 together with an indication when samples were taken for analysis. Full details are given in Dunstan *et al.* (1993).



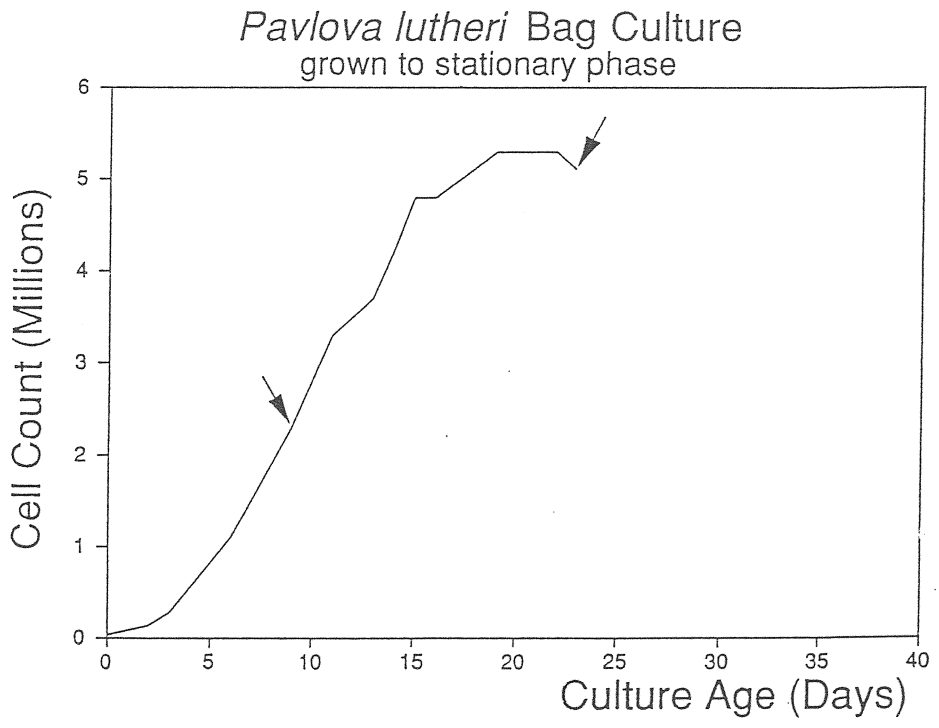


Figure 8. Changes in the number of cells per ml in 100 litre bag cultures of *Pavlova lutheri*. The first set was harvested semi-continuously while the second was grown to stationary phase. Arrows indicate when samples were taken for lipid analysis.

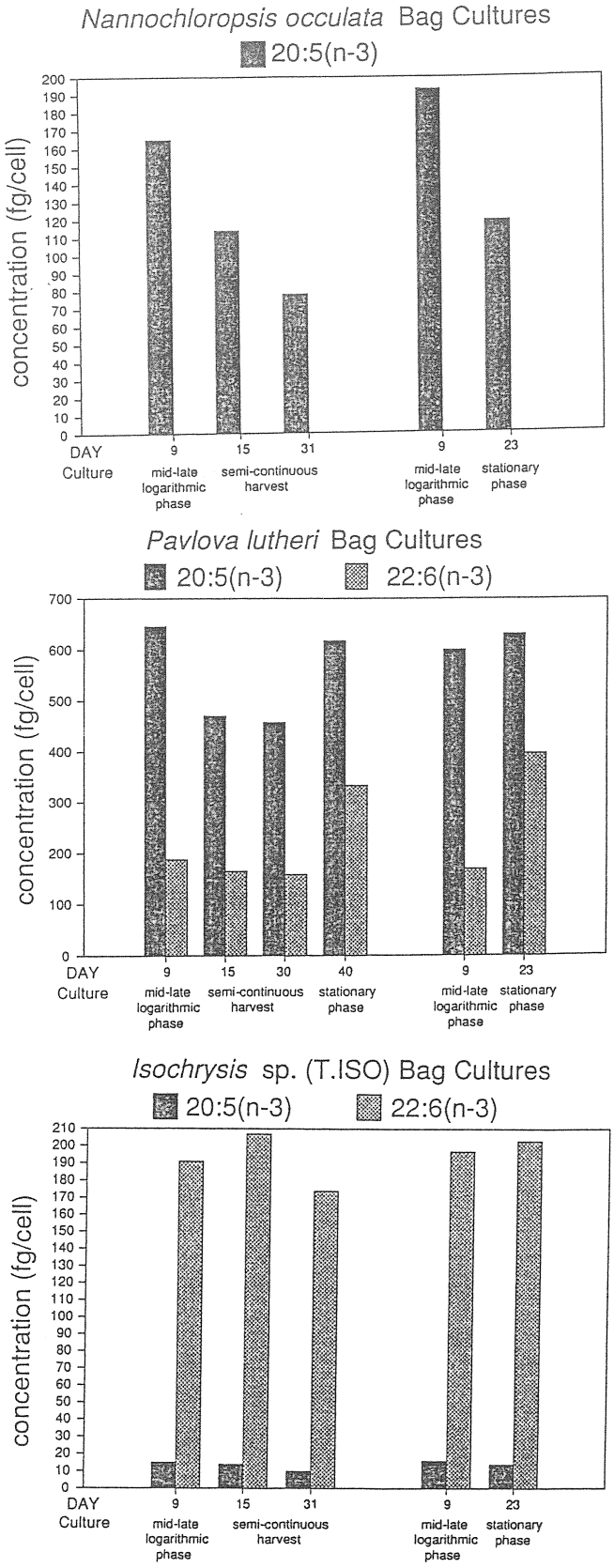


Figure 9. Concentrations of 20:5(n–3) and 22:6(n–3) in 3 species of microalgae grown in 100 litre bag culture and harvested at different times. Data from 2 experiments are shown. The first where the culture was harvested semi–continuously (see text) and the second where the culture was grown to stationary phase.

Changes in the microalgal cultures harvested semi-continuously			
Species	*Pavlova lutheri*	*Nannochloropsis occulata*	*Isochrysis* sp. (T.ISO)
PUFA (fg/cell)	↓	↓	–
PUFA (% of FAME)	↓	↓	–
Triacylglycerols	–	–	–
total cell mass dry wt/cell	↓	↓	⇑

Changes in the microalgal cultures grown to stationary phase			
Species	*Pavlova lutheri*	*Nannochloropsis occulata*	*Isochrysis* sp. (T.ISO)
PUFA (fg/cell)	⇑	↓	–
PUFA (% of FAME)	↓	↓	↓
Triacylglycerols	⇑	⇑	⇑
total cell mass dry wt/cell	↓	↓	⇑

increase	⇑
decrease	↓
no change	–

Figure 10. Summary of the major changes observed in PUFA and triacylglycerol content in 3 species of microalgae harvested semi-continuously or grown to stationary phase in comparison with the composition of cells harvested at mid–late exponential phase.

Abundances of the major long–chain PUFA in the 3 species expressed as femtogram per cell are shown in Figure 9. A summary of the major changes observed is provided in Figure 10. None of the samples of *Nannochloropsis oculata* contained 22:6(n–3), and the amount of 20:5(n–3) was very much higher in mid to late logarithmic phase (Figure 11). Stationary phase cultures of all three species showed increased proportions (%) and cellular contents of triacylglycerols, and saturated and monounsaturated fatty acids with correspondingly decreased proportions of polar lipids and most PUFA relative to logarithmic phase cultures (Table 14). The exception was the proportion and cellular content of 22:6(n–3) in *P. lutheri* which increased with triacylglycerol content. The cellular content of PUFA in cultures of *Isochrysis* sp. did not change significantly with culture technique or growth phase (Table 15). The mass of long–chain (n–3) PUFA per volume of culture was significantly higher in stationary phase cultures due to the higher cell counts per volume. These findings are summarised in Figure 10. Clearly, PUFA production by microalgae can be manipulated which could lead to improved animal growth and a reduction of the production costs in mariculture operations. Large scale culture and harvesting of microalgae for PUFA content may also be of value to the biotechnology industry.

For mariculture operations animals are often fed rations based on cell counts per volume. In order to minimise the number of microalgal cells needed to achieve the same PUFA ration per animal, it is desirable to maximum PUFA content per cell. To achieve this in the species examined, our data suggest that logarithmic phase batch cultures of *N. oculata* and stationary phase cultures of *P. lutheri* should be harvested. Because there was little effect of growth phase or culture technique on the cellular contents of PUFA in *Isochrysis* sp., harvest time in this species would be less important with respect to cellular contents of PUFA. Due to significant diurnal variations in the production and turnover of triacylglycerols, glycolipids and fatty acids in some species of microalgae, PUFA content can further be optimised by harvesting at particular times of the day (e.g. Sukenik and Carmeli, 1990).

For mariculture and biotechnology operations in which the production of PUFA is specified according to culture volume, our data indicate that stationary phase cultures (with the highest cell counts per volume) produced significantly higher amounts of the (n–3) PUFA per litre of culture than logarithmic phase cultures. It should be noted, however, that the total yield of PUFA over the entire life of the culture also needs to be considered. For example, combined harvests from the semi–continuous cultures (which can be sustained and cropped for several weeks), should yield more of the (n–3) PUFA in total than a single cropping of the relatively lipid and (n–3) PUFA rich stationary phase batch cultures. This is even though at any one time, the former contains lower amounts of these PUFA per culture volume than the latter.

If the lipid class to which fatty acids are esterified or the overall proportions or amounts of fatty acids in the dietary microalgae are considered to be nutritionally important to the species of animal being intensively reared (e.g. due to differential assimilation of

fatty acids), the culture growth phase may determine the optimum time for harvest. Harvesting any of the relatively lipid–rich stationary phase cultures examined, results in proportionally less polar lipid and PUFA in the microalgal cells due to the increased proportions of triacylglycerol and saturated and monoenoic fatty acids. The nutritional significance of which, should be evaluated for the particular animal being reared.

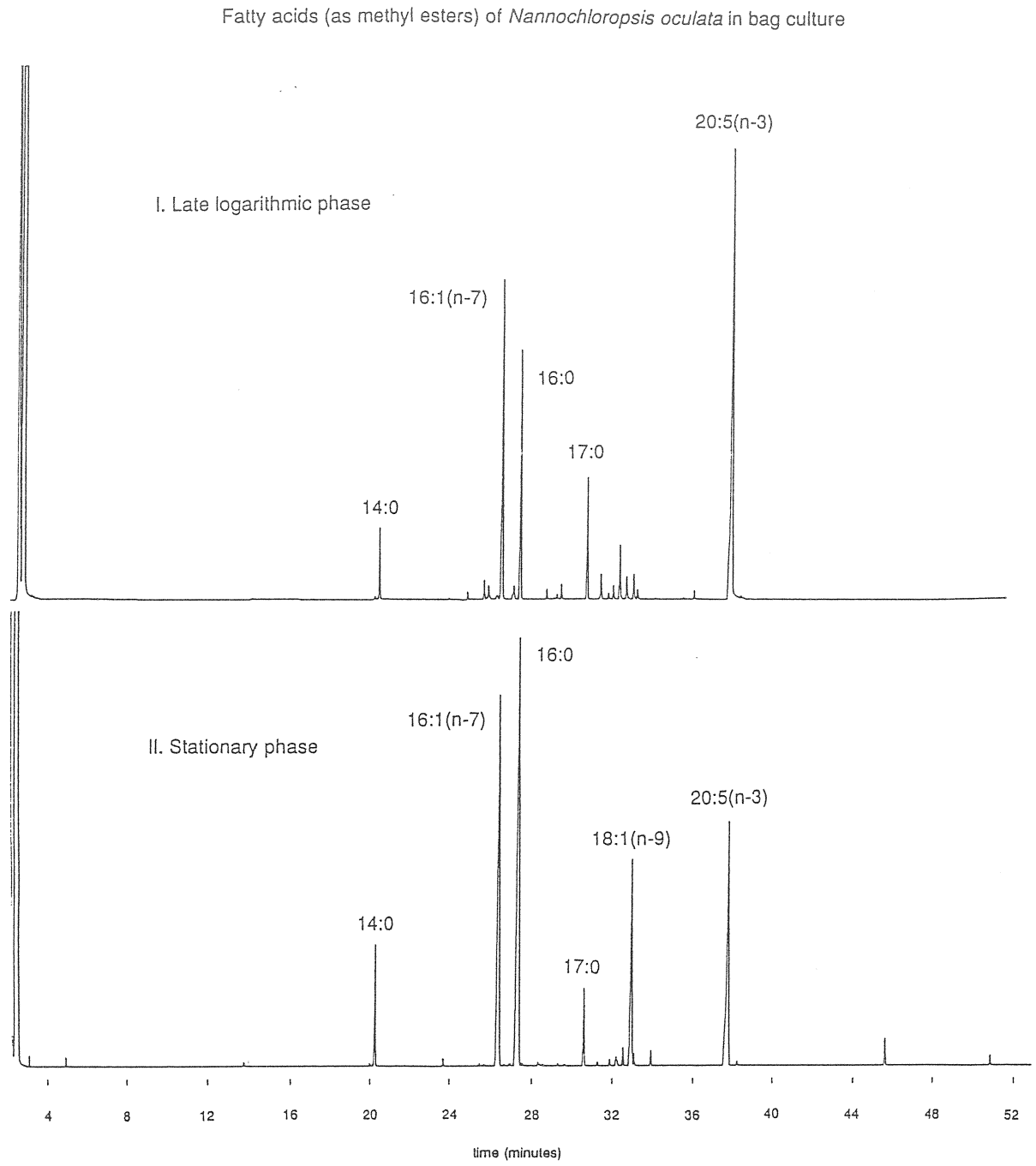


Figure 11. Fatty acids as methyl esters in large–scale bag cultures of *Nannochloropsis oculata* grown to late logarithmic and stationary phase. Note the absence of 22:6(n–3) in both samples, and much higher abundance of 20:5(n–3) in the culture grown to late logarithmic phase.

Table 14. Proportions (%) of major solvent-extractable lipid classes and total cellular lipid content (pg cell^{-1}) in three species of microalgae grown in mass culture, sampled at different growth phases and cultured by different techniques.

culture phase	culture technique	culture age (days)	number of replicate cultures	average cell count at sampling (x10^6 cells ml^{-1})	Lipid class (%) PL	SD	ST	4ME	FFA	TG	HC	MK	EK	total cellular lipid content (pg cell^{-1}±range)
Nannochloropsis oculata														
logarithmic	batch	9	4	13.4	90	–	3.9	–	2.4	1.9	1.4	–	–	0.9±0.10
logarithmic	semicontinuous	15	2	21.3	83	–	5.7	–	2.1	7.0	2.2	–	–	0.5±0.14
logarithmic	semicontinuous	31	2	21.9	85	–	5.9	–	3.1	3.0	2.5	–	–	0.5±0.04
stationary	batch	17	1	–	71	–	5.3	–	tr	22	2.2	–	–	0.7
stationary	batch	23	2	24.3	51	–	4.6	–	0.5	41	3.1	–	–	1.1±0.05
Pavlova lutheri														
logarithmic	batch	9	3	2.33	88	4.7	2.9	1.4	3.1	tr	–	–	–	5.0±0.74
logarithmic	semicontinuous	15	2	3.72	84	5.4	2.9	1.5	1.2	4.9	–	–	–	5.3±0.66
logarithmic	semicontinuous	30	2	2.97	87	5.2	2.5	1.5	1.5	2.4	–	–	–	5.1±0.64
stationary	batch	40	2	3.54	50	5.9	5.0	2.0	1.0	35	0.5	–	–	8.2±0.19
stationary	batch	23	1	5.06	57	6.2	4.5	2.5	1.2	28	0.7	–	–	7.0
Isochrysis sp.														
logarithmic	batch	9	4	3.53	83	–	1.2	–	–	0.6	–	11	3.8	4.8±0.54
logarithmic	semicontinuous	15	2	4.07	72	–	0.7	–	–	11	–	14	3.2	6.1±0.73
logarithmic	semicontinuous	31	2	4.42	74	–	0.8	–	–	4.8	–	16	4.2	4.9±0.05
stationary	batch	23	2	7.25	54	–	0.8	–	–	20	–	22	4.1	7.0±0.92

PL, polar lipids and chlorophylls; SD, steroidal diols (tentative identification); ST, 4-desmethyl sterols; 4ME, 4-methyl sterols; FFA, free fatty acids; TG, triacylglycerols; HC, hydrocarbons; MK, C_{37}-C_{38} methyl ketones; EK, C_{38}-C_{39} ethyl ketones; other minor components (less than 0.7%) not included in the Table are included in the total.
tr: less than 0.5%

Table 15. Proportions (%) of the major fatty acids, total cellular content of fatty acids (pg cell^{-1}) and "essential" PUFA production per litre of culture in three species of microalgae grown in mass culture, sampled at different growth phases and cultured by different techniques.

	Nannochloropsis oculata					*Pavlova lutheri*					*Isochrysis* sp.			
	logarithmic			stationary		logarithmic			stationary		logarithmic			stationary
	batch	semicontinuous		batch		batch	semicontinuous		batch		batch	semicontinuous		batch
age (days)	9	15	31	17	23	9	15	30	40	23	9	15	31	23
replicates	4	2	2	1	2	3	2	2	2	1	4	2	2	2
Saturated Fatty Acids														
14:0	5.0	5.4	4.8	5.8	5.4	8.8	9.2	10.0	7.9	8.1	18.4	16.6	13.9	16.0
i15:0	0.3	0.5	0.5	0.1	tr[1]	0.1	tr	–[2]	–	0.1	tr	–	–	0.1
15:0	0.3	0.3	0.3	0.4	0.4	0.2	0.2	0.2	0.3	0.2	0.4	0.3	0.2	0.3
16:0	16.4	22.7	20.3	27.7	33.3	17.3	21.4	20.8	23.4	22.8	9.3	11.5	12.6	14.1
18:0	0.3	0.4	0.3	0.4	0.6	0.3	0.3	0.2	0.3	0.3	0.1	0.2	0.1	0.4
subtotal	22.3	29.3	26.2	34.4	39.7	26.7	31.1	31.2	31.9	31.5	28.2	28.6	26.8	30.9
Monounsaturated Fatty Acids														
16:1(n–7)	21.8	19.7	21.2	19.6	24.4	14.4	18.9	19.9	23.0	20.9	5.0	2.9	3.2	2.9
16:1(n–5)	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.2	tr	–	–	tr
16:1(n–13)t	1.0	0.8	0.9	0.4	0.2	–	–	–	–	–	0.1	tr	0.1	tr
17:1(n–8)	0.2	0.1	0.2	0.1	0.2	–	–	–	–	–	0.2	0.1	0.2	0.1
18:1(n–9)	2.1	5.3	4.1	9.0	11.9	0.6	0.6	0.5	1.3	0.8	7.6	16.7	15.1	25.6
18:1(n–7)	0.6	0.6	0.5	0.4	0.5	2.4	3.2	3.3	3.3	3.4	2.3	0.9	1.7	1.4
subtotal	25.8	26.6	27.0	29.6	37.3	17.5	22.9	23.8	27.8	25.3	15.2	20.6	20.3	30.0

(continued next page)

Table 15 (continued).

	Nannochloropsis oculata					*Pavlova lutheri*					*Isochrysis* sp.			
	logarithmic			stationary		logarithmic			stationary		logarithmic			stationary
	batch	semicontinuous		batch		batch	semicontinuous		batch		batch	semicontinuous		batch
age (days)	9	15	31	17	23	9	15	30	40	23	9	15	31	23
replicates	4	2	2	1	2	3	2	2	2	1	4	2	2	2
Polyunsaturated Fatty Acids														
16:2(n–7)	1.0	0.5	0.4	0.2	0.1	2.1	0.7	1.1	0.7	0.5	1.4	0.8	0.3	0.6
16:2(n–4)	0.5	0.2	0.1	0.1	0.1	0.7	0.6	0.7	0.5	0.6	0.6	0.5	0.5	0.5
18:2(n–6)	1.7	1.7	1.8	1.3	0.9	0.6	0.5	0.4	0.8	0.5	3.7	4.5	4.5	3.7
18:3(n–6)	0.9	0.5	1.0	0.4	0.4	0.5	0.2	0.2	0.2	0.1	2.0	1.4	0.7	0.3
18:3(n–3)	0.1	0.1	tr	0.1	tr	2.1	1.1	1.1	0.6	0.6	5.7	4.7	5.8	3.9
18:4(n–3)	–	–	–	–	–	10.9	7.9	6.6	4.9	6.2	26.8	24.2	25.4	17.1
18:5(n–3)	–	–	–	–	–	–	–	–	–	–	2.5	1.4	0.3	0.7
20:4(n–6)	6.1	6.7	7.4	6.3	3.9	0.7	0.6	0.6	0.6	0.5	0.1	tr	tr	0.1
20:5(n–3)	39.8	33.4	34.7	25.9	16.4	27.6	23.1	22.9	17.0	17.7	0.8	0.6	0.6	0.5
22:5(n–6)	–	–	–	–	–	0.9	1.1	0.9	1.5	1.6	1.4	1.9	2.3	1.4
22:5(n–3)	–	–	–	–	–	–	0.1	0.1	0.8	0.9	tr	tr	0.1	–
22:6(n–3)	–	–	–	–	–	7.9	8.1	7.9	9.2	11.2	9.5	9.0	10.2	6.8
subtotal	50.1	43.1	45.4	34.3	21.8	54.0	44.0	42.5	36.8	40.4	54.5	49.0	50.7	35.6
others (3)	1.8	1.0	1.4	1.7	1.2	1.8	2.0	2.5	3.5	2.8	2.1	1.8	2.2	3.5
Total cellular fatty acid content														
(pg cell^{-1})	0.45	0.34	0.28	0.43	0.75	2.27	2.04	2.01	3.63	3.56	2.04	2.30	1.70	2.99
PUFA production per litre of culture														
mg 20:5(n–3)/l	2.45	2.42	2.12	3.63	2.95	1.45	1.75	1.37	2.20	3.19	0.05	0.06	0.04	0.10
mg 22:6(n–3)/l	–	–	–	–	–	0.42	0.61	0.48	1.18	2.01	0.69	0.84	0.77	1.48

(1) tr: less than 0.05%; (2) nd: none detected; (3) sum of less abundant (less than 1%) and unidentified fatty acids

5.2.2 Effects of irradiance

Summary

A study was conducted to determine how the fatty acid composition of *Isochrysis* sp. (T.iso) changes with light intensity and a detailed account of these data was published (Brown *et al.*, 1993). At low light intensities (50 and 100 $\mu E.m^{-2}.sec^{-1}$) relatively more C_{18} PUFA was produced, whereas at 500 and 1000 $\mu E.m^{-2}.sec^{-1}$ (typical of shaded tropical aquaculture ponds during the day) there was an increased production of the essential fatty acid 22:6(n–3). Large proportions of long–chain unsaturated ketones (16 to 21% of the total lipid) were detected in all samples, although there was no significant changes in the proportions of the various lipid fractions. The dietary significance of these ketones in mariculture feeds has not yet been established. It has been suggested they may act as antioxidants and help in preserving feeds during storage, but there is no direct evidence for this. Anecdotal evidence suggests that increased tank fouling may occur as a result of increased production of faeces.

Axenic cultures of *Isochrysis* sp. (T.Iso) were grown at different irradiance levels to examine the effect of light intensity on lipid and fatty acid composition. The total lipid contents per cell of *Isochrysis* sp. (T.Iso) cultures (Table 16) are comparable to results for species of *Isochrysis* grown under different ranges of irradiance level and light conditions (Claustre and Gostan, 1987; Harrison *et al.*, 1990; Thompson *et al.*, 1990; Renaud *et al.*, 1991; Sukenik and Wahnon, 1991). A significant proportion of the total lipid (16.1–20.8%) consisted of very long–chain ($>C_{36}$) unsaturated alkenones and C_{36} fatty acids (Table 16). The work of Harrison *et al.* (1990) and Thompson *et al.* (1990), like the present study showed no significant difference in lipid per cell as continuous irradiance level is increased (Table 16), although in their study, irradiance levels up to only 225 $\mu E.m^{-2}.sec^{-1}$ were examined. Renaud *et al.* (1991) found increase in lipid levels with 12:12 light:dark irradiance, although their results from pond culture show higher average lipid levels of 10.3 $pg.cell^{-1}$ when grown at 1200 $\mu E.m^{-2}.sec^{-1}$ compared with 7.1 and 7.2 $pg.cell^{-1}$ at 390 and 620 $\mu E.m^{-2}.sec^{-1}$.

Claustre and Gostan (1987) found approximately 30% higher concentrations of total lipid per cell in *I. galbana* at higher continuous irradiance (400 $\mu E.m^{-2}.sec^{-1}$), whereas Sukenik and Wahnon (1991) reported approximately 30% lower lipid per cell at increased continuous irradiance (500 $\mu E.m^{-2}.sec^{-1}$) compared to lipid levels in cultures grown at 25 and 30 $\mu E.m^{-2}.sec^{-1}$ respectively. The concentration per cell and composition of the various lipid classes (polar lipids, sterols, free fatty acids, and very long–chain methyl and

ethyl alkenones) in *Isochrysis* sp. (T.Iso) grown at different levels of irradiance remained relatively constant in our study, but the abundance of the very long–chain 36:2 methyl ester increased with irradiance level. Triacylglycerols did not increase above trace levels at any of the irradiance levels examined.

Higher concentrations per dry weight of total lipid, fatty acids and very long–chain unsaturated lipids were evident at lower irradiance levels (50 and 100 $\mu E.m^{-2}.sec^{-1}$), largely due to decreased cell dry weight. The lower cell dry weight at reduced irradiance levels was not due to reduced levels of lipid, carbohydrate, protein or pigment. Thompson *et al.* (1990) demonstrated reduced cell volume and carbon quota in *Isochrysis* sp. (T.Iso) grown at much lower irradiance levels (down to 14 $\mu E.m^{-2}.sec^{-1}$), while Claustre and Gostan, (1987) noted that the cell volume of the prymnesiophyte *H. elongata* decreased at extremely low light levels (25 $\mu E.m^{-2}.sec^{-1}$), whereas in *I. galbana* there was an initial decrease in cell volume when irradiance level was reduced from 400 to 25 $\mu E.m^{-2}.sec^{-1}$, but over the next sixty hours the cells returned to their original volume.

Major fatty acids were 14:0, 16:0, 16:1(n–7), 18:1(n–9), 18:3(n–3), 18:4(n–3), 18:5(n–3) and 22:6(n–3) (Table 17). In cultures grown at lower irradiance level (50 and 100 $\mu E.m^{-2}.sec^{-1}$) there were proportionally higher levels of the shorter chain PUFA 18:3(n–3) and 18:4(n–3), whereas at the higher irradiance level (500 and 1000 $\mu E.m^{-2}.sec^{-1}$) cultures contained higher proportions of 22:6(n–3). Although there was an increase in the proportion of total PUFA at low irradiance level, the proportion of the "essential fatty acid" 22:6(n–3) was diminished. There was very little change in total lipid and fatty acid concentrations between the different light regimes when expressed on a per cell basis, although higher concentrations of fatty acids per dry matter were evident at low irradiance level, due to a decrease in cell dry matter.

Prymnesiophytes generally have very low proportions of C_{16} PUFA and 20:5(n–3), significant proportions of 14:0, 16:0, 16:1(n–7), 18:3(n–3), 18:4(n–3), the long–chain essential PUFA, 22:6(n–3), and produce the unusual fatty acid 18:5(n–3) (Table 17). In the present study, lower irradiance level (50 and 100 $\mu E.m^{-2}.sec^{-1}$) cultures of *Isochrysis* sp. (T.iso) had reduced proportions of 16:0 and 18:1(n–9) and higher proportions of the shorter chain PUFA 18:3(n–3) and 18:4(n–3), whereas the higher irradiance level (500 and 1000 $\mu E.m^{-2}.sec^{-1}$) cultures contained higher proportions of 22:6(n–3) (Table 17). Similar variations in fatty acid composition over comparable ranges of irradiance level were reported by Renaud *et al.* (1991) and Sukenik and Wahnon (1991).

Higher irradiance level (up to 300 $\mu E.m^{-2}.sec^{-1}$) generally increases growth rate in this species (Sukenik and Wahnon, 1991) which can result in a more rapid onset of stationary phase in cultures. Any comparison of variation in fatty acid distribution between different studies can be complicated by the fact that lipid composition changes considerably as cells enter stationary phase. In other experiments, we have observed that the amount of triacylglycerols increases from trace amounts to over 20 % of the total lipid in stationary cultures of *Isochrysis* sp. (T.Iso) (unpublished data). In *Isochrysis* sp. (T.Iso) the

triacylglycerol fatty acids are predominantly 16:0 and 18:1(n–9) (Sukenik and Wahnon, 1991). On a per cell basis, the total concentration of these fatty acids increased two and five–fold respectively in stationary phase cultures, with a correspondingly reduced proportion of fatty acids such as PUFA, which are predominantly bonded to polar lipids such as galactolipids and phospholipids.

All of the cultures of *Isochrysis* sp. in the present study were harvested at late–logarithmic phase and had similar low lipid concentrations and no more than trace amounts of triacylglycerols. Thus, the changes in PUFA proportions observed [i.e. higher proportions of 22:6(n–3) at high irradiance levels with a simultaneous decrease in shorter chain PUFA and *vice versa* at low irradiance levels] were probably induced by irradiance level and not the result of a more rapid onset of stationary phase in the cultures. The lower light levels were probably responsible for the increased proportion of 18:4(n–3), because the 18:4(n–3)–rich galactolipids are a major components of photosynthetic membranes (Sukenik and Wahnon, 1991). At higher irradiance levels this 18:4(n–3) may be chain elongated and desaturated to be accumulated as 22:6(n–3) in the phospholipids.

In cultures of *Isochrysis* aff. *galbana* (T.iso), Thompson *et al.* (1990) found higher proportions of saturated fatty acids and significantly lower proportions of 18:1(n–9) and PUFA at extremely low irradiance levels (14 $\mu E.m^{-2}.sec^{-1}$) compared with cultures grown at 44 and 225 $\mu E.m^{-2}.sec^{-1}$, with maximum levels of PUFA at 125 $\mu E.m^{-2}.sec^{-1}$. Harrison *et al.* (1990) have suggested that these extremely low levels of 22:6(n–3) are due to its "redeployment" to 14:0, although it seems more likely to us that 22:6(n–3) and the other PUFA are not produced by the alga at this extremely low irradiance level.

In *Isochrysis* sp. (T.Iso) neither the total concentrations nor the concentrations of the individual very long–chain unsaturated lipids varied significantly with irradiance level. The exception was at 50 $\mu E.m^{-2}.sec^{-1}$, where the total concentration per cell of very long–chain lipids was lower, but the proportions of individual components did not change significantly. The degree of unsaturation and hence ratios of the individual components varies with ambient temperature in another prymnesiophyte, *Emiliania huxleyi* (Brassell *et al.*, 1986; Prahl and Wakeham, 1987), but the total amounts are not affected (Prahl *et al.*, 1988).

The variation of the lipid and fatty acid composition of the diatom *Thalassiosira pseudonana* with growth phase as well as light regime and light intensity was also examined (Brown *et al.*, 1995b). In samples of this species harvested at stationary phase, the proportion of PUFA was much less (as with the large scale culture experiments), due primarily to the increase of storage lipid within the cells. The cultures exposed to more light (whether due to increased duration or intensity) accumulated more storage lipid and hence lower proportions of PUFA. Protein was highest in cells grown to logarithmic phase, whereas carbohydrate contents were particularly affected by the light regime. The amino acid profile of the cells were similar in all experiments irrespective of harvest stage or light regime.

Table 16. Percent composition of lipid classes and total lipid concentration in *Isochrysis* sp. (T.Iso) grown under different irradiance levels

Irradiance $\mu E.m^{-2}.sec^{-1}$	FAE & EA	MA	FFA	ST	PIG	PL	Total Lipid $pg.cell^{-1}$	Total Lipid $mg.g^{-1}$ dry wt
50	3.9	13.0	4.2	0.9	3.3	74.6	4.46	14.8
100	3.5	12.6	2.5	0.7	2.1	78.5	5.20	11.6
250	4.1	14.1	4.1	0.8	1.7	74.2	4.89	8.60
500	5.0	12.8	4.0	1.5	1.8	74.9	5.37	8.65
1000	6.4	14.4	4.2	0.7	1.4	73.0	5.49	10.3

FAE and EA esters of 36:2 fatty acid and ethyl alkenones; MA methyl alkenones; FFA free fatty acids; ST, sterols; PIG unidentified peak including pigments; PL, polar lipids and chlorophylls.

Table 17. Relative fatty acid composition (% of total fatty acids) of *Isochrysis* sp. (T.Iso) cultures grown at different irradiance levels

Irradiance ($\mu E.m^{-2}.sec^{-1}$)	50	100	250A	250B	500	1000
Saturated fatty acids						
14:0	17.8	17.0	17.0	17.4	17.3	16.5
15:0	0.8	0.4	0.9	0.8	1.2	0.8
16:0	10.5	11.4	13.3	13.3	12.1	12.8
18:0	0.2	0.3	0.5	0.3	0.2	0.2
subtotal[1]	29.5	29.6	32.4	32.4	31.3	30.9
Monounsaturated fatty acids						
16:1(n-9)	0.1	0.2	0.1	0.1	0.1	0.2
16:1(n-7)	5.0	4.9	5.8	5.6	5.5	5.1
17:1(n-8)	0.3	0.1	0.2	0.2	0.4	0.2
18:1(n-9)	7.3	8.5	11.4	11.6	10.6	10.7
18:1(n-7)	1.1	1.1	1.4	1.5	1.5	1.4
subtotal[1]	13.9	14.9	19.2	19.2	18.2	17.7
Polyunsaturated fatty acids						
16:2(n-7)	0.8	1.1	1.1	1.1	1.0	1.1
16:2(n-4)	0.3	0.2	0.3	0.3	0.2	0.2
16:3(n-4)	0.3	0.3	0.2	0.2	0.2	0.2
18:2(n-6)	2.5	1.9	1.6	1.7	1.5	1.8
18:3(n-6)	0.7	0.5	0.3	0.3	0.2	0.2
18:3(n-3)	6.2	5.5	4.8	5.0	4.2	4.2
18:4(n-3)	24.9	26.0	20.1	20.6	19.4	19.1
18:5(n-3)	5.9	5.6	4.7	4.8	5.7	5.9
20:5(n-3)	0.4	0.4	0.4	0.4	0.5	0.3
22:5(n-6)	1.7	1.5	1.5	1.5	1.7	1.9
22:6(n-3)	12.5	12.3	12.9	12.0	15.0	15.8
subtotal[1]	56.6	55.5	48.4	48.4	50.2	51.2
unidentified	tr	tr	tr	tr	0.2	0.2
Total percent	100.0	100.0	100.0	100.0	100.0	100.0
Total concentration of fatty acids						
$pg.cell^{-1}$	2.22	2.36	1.88	1.95	1.95	2.07
$mg.g^{-1}$ dry wt	73.7	52.5	33.2	34.4	31.5	38.7

Includes contributions from minor components not listed; tr, trace amounts detected (less than 0.5%); A and B are replicate extracts of the same culture

5.3. Studies with the oyster Crassostrea gigas (Objective 4)

Summary

Studies of the lipid class compositions of adult Pacific oysters [*Crassostrea gigas* (Thunberg)] were determined using Iatroscan TLC–FID, to validate our methods prior to carrying out feeding experiments. Adult oysters from two different size classes showed high contents of triacylglycerols and essential PUFA (over 35% of total fatty acids). There was very little difference in the proportions of lipid classes, fatty acids or sterols.

However, to our surprise, significant differences in some of the lipid components were evident according to the method of sample preparation used prior to lipid extraction with chloroform–methanol solvent mixtures (Dunstan *et al.*, 1993a). Lyophilization (freeze drying) of the oyster tissue led to a significant reduction in the amounts of triacylglycerols extracted by solvents in three separate experiments (7.0, 54.0 and 52.5% extracted), but there was no significant change in the amounts of polar lipids, total sterols, free fatty acids or hydrocarbons between lyophilized and non–lyophilized samples (Table 18). After some trial experiments, we discovered that we had to add water to the freeze–dried samples prior to lipid extraction to get satisfactory yields of triacylglycerols (>91%). It should be pointed out that lyophilization is commonly used in many laboratories to prepare samples for extraction and it is clear that, at least with oysters, this can lead to greatly underestimated yields of lipids and fatty acids.

Fatty acid compositions were determined by capillary GC analysis of methyl ester derivatives and by GC–MS analysis of 4,4–dimethyloxazoline (DMOX) derivatives. The major esterified fatty acids were 16:0, 20:5(n–3) and 22:6(n–3). C_{20} and C_{22} non–methylene interrupted (NMI) fatty acids comprised 4.5 to 6.0 % of the total fatty acids. The NMI trienoic fatty acid 22:3(7,13,16) was also identified. Lyophilized samples had less 16:0 and C_{18} unsaturated fatty acids whereas C_{20} and C_{22} unsaturated fatty acids comprised a higher proportion of the total fatty acids (Table 19). Sterol distributions were complex and showed a strong influence of dietary–derived components (Table 20).

Species of true oyster (Family Ostreidae), primarily of the genera *Crassostrea* and *Ostrea* are grown in intensive mariculture farms for human consumption world–wide. The

high economic value of these bivalve molluscs has led to considerable efforts to improve production by increasing survival and growth rates, especially of the larval stages. Insufficient quantities of the long-chain polyunsaturated fatty acids 20:5(n-3) and 22:6(n-3) in the diet can lead to reduced growth rates and increased mortality in larval and juvenile bivalve molluscs (e.g. Trider and Castell, 1980; Langdon and Waldock, 1981; Enright *et al.*, 1986a). These acids were abundant in the samples analysed here (>25% of total fatty acids; Figure 12 and Table 19). Unusual C_{20} and C_{22} non-methylene interrupted (NMI) fatty acids comprised 4.5 to 5.9% of the total fatty acids; the NMI trienoic fatty acid 22:3(*7,13,16*) was also identified. These NMI fatty acids are not found in the oyster's diet, but are presumed to be synthesized by the oyster from dietary fatty acids *via* a Δ^5 desaturase and chain-elongation.

Adult Pacific oysters [*Crassostrea gigas* (Thunberg)] were obtained from Shellfish Culture Pty. Ltd. from Pipe Clay Lagoon, Tasmania (Experiment 1), and from Mures Fish restaurant (Experiment 2). The lipid class compositions (Table 18) were examined using Iatroscan TLC/FID and fatty acid compositions determined by GC/MS. The fatty acid methyl esters were separated using argentation TLC and also analyzed as their 4,4-dimethyloxazoline (DMOX) derivatives using GC/MS. Capillary GC traces showing the distributions of fatty acids analysed on both polar and non-polar columns are shown in Figure 12. Quantitative data for fatty acids and sterols are given in Tables 19 and 20.

Very little difference was found in the proportions of the various lipid fractions, fatty acids or sterols between samples of adult oysters of two different sizes. However, significant differences in some of the lipid components were evident according to the method of sample preparation used prior to lipid extraction with solvents (Table 18). Lyophilization (freeze drying) of samples led to a significant reduction in the amounts of triacylglycerols extracted by solvents in two separate experiments (7.0 and 52.5% extracted). Extracts from lyophilized samples had less 16:0, C_{18} unsaturated fatty acids (Table 19), and 24-ethylcholest-5-en-3β-ol (Table 20), while C_{20} and C_{22} unsaturated fatty acids comprised a higher proportion of the total fatty acids. There was no significant change in the amounts of polar lipids, total sterols, free fatty acids or hydrocarbons observed in extracts from lyophilized samples relative to extracts from non-lyophilized samples. Addition of water to the freeze-dried samples prior to lipid extraction greatly improved lipid yields and resulted in most of the triacylglycerols being extracted.

Larval viability has also been related to the total lipid content of oyster eggs and larvae (Helm *et al.*, 1973; Gallager *et al.*, 1986), and variations in total lipid with age and season in adult oysters gives a measure of the mollusc's nutritional and reproductive status (Trider and Castell, 1980; Ruiz *et al.*, 1992). As in most marine animals, the polar lipids in species of *Crassostrea* contain relatively more PUFA than neutral lipids (which are primarily triacylglycerols) (Langdon and Waldock, 1981; Watanabe and Ackman, 1974; Waldock and Nascimento, 1979). These lipid fractions are not homogeneous with respect to fatty acid composition, so incomplete lipid extraction could result in incorrect estimates of total lipid content and polyunsaturated fatty acid composition and hence of larval condition and adult reproductive status in cases where such lipid indices are used.

Table 18. Concentration (mg/g wet wt.) of lipid classes, total lipid (%) and moisture content in adult *Crassostrea gigas* tissues which had been (i) frozen ("control") or (ii) frozen and lyophilized or (iii) lyophilized and rehydrated

	Replicate	*Lipid class (mg/g wet wt.)*[a]					*Total lipid*		Water
		PL	ST & DG	FFA	TG	HC & WE	% wet wt.	% dry wt.	content %
Experiment 1									
"Control"–large[b]		9.1	0.7	0.2	11.3	tr[e]	2.1	ND[f]	ND
"Control"–small		9.9	0.9	0.4	11.5	tr	2.3	10.2	77.6
Lyophilized (42h)[c]–large		9.8	1.2	0.4	0.9	0.1	1.2	6.4	80.6
Lyophilized (42h)–small		8.6	1.0	0.1	0.7	tr	1.1	6.2	83.2
Experiment 2									
"Control"	A	6.3	1.4	1.5	5.8	0.8	1.6	nd	nd
	B	7.1	1.5	1.6	6.0	1.0	1.7	nd	nd
Lyophilized (24h)	A	6.4	1.5	1.4	3.3	0.6	1.3	7.1	81.7
	B	6.8	1.5	1.4	3.1	0.6	1.3	7.3	81.7
Lyophilized–rehydrate[d] (24h)	A	6.4	1.4	1.6	5.2	0.8	1.5	7.9	80.6
	B	6.6	1.4	1.7	5.7	0.9	1.6	8.6	81.1

[a] PL, polar lipids; ST & DG, sterols plus diacylglycerols; FFA free fatty acids; TG, triacylglycerols; HC & WE, hydrocarbons plus wax esters.

[b] "Control", solvent extracted lipids of non–lyophilized sample; large, mean soft tissue weight 8.9±0.5 g; small, mean soft tissue weight 5.8±0.4 g.

[c] Lyophilized, solvent extracted lipids of freeze dried sample; times (h) refer to duration of lyophilization.

[d] Lyophilized–Rehydrate, solvent extracted lipids of freeze dried sample after rehydration with water.

[e] tr, trace = <0.1 mg/g wet wt.

[f] ND not determined.

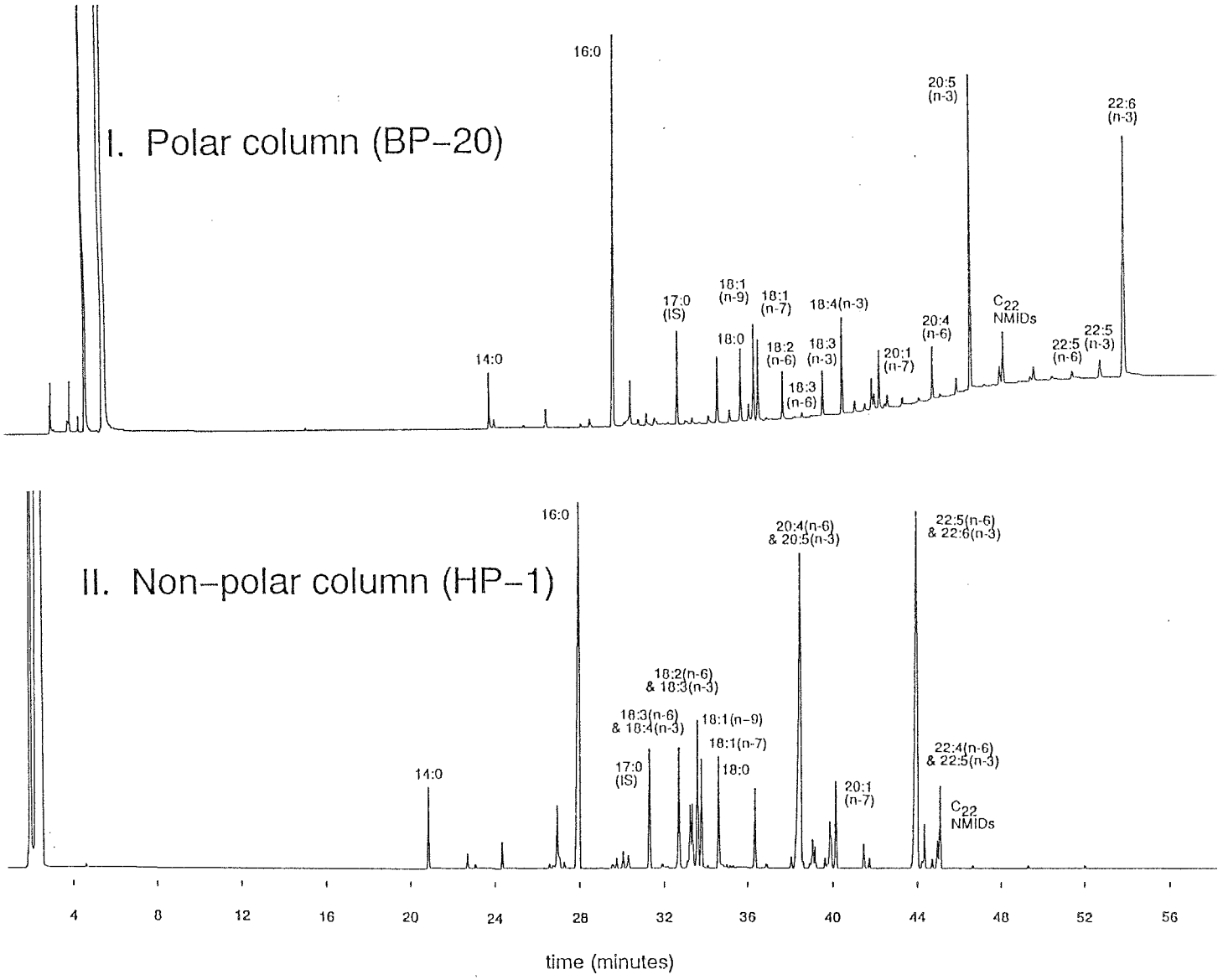


Figure 12. Total fatty acids in oysters (*Crassostrea gigas*) analysed on polar and non-polar gas capillary columns.

Table 19. Percentage composition of the fatty acids in adult *Crassostrea gigas*

	"Control" [a]		*Lyophilized* [b]	
Fatty acids	Large[c]	Small	Large	Small
Saturated Fatty Acids				
14:0	2.3	2.3	1.4	1.4
15:0	0.8	0.8	0.6	0.7
16:0	18.2	18.6	15.6	15.4
18:0	3.4	3.5	4.0	3.7
19:0	0.1	0.1	0.1	0.1
20:0	0.1	0.1	0.1	0.1
22:0	0.1	0.1	0.1	0.1
24:0	0.2	0.1	0.1	0.1
Subtotal[d]	25.2	25.6	21.9	21.5
Branched-chain Fatty Acids				
4,8,12 TMTD	0.5	0.6	0.8	1.0
*i*15:0	0.2	0.2	0.2	0.1
*i*16:0	0.2	0.2	0.1	0.2
*i*17:0	0.5	0.5	0.5	0.4
*a*17:0	0.1	0.1	0.2	0.2
br18:0	0.4	0.4	0.4	0.4
*i*18:0	0.1	0.1	0.1	0.2
phytanic acid	0.1	0.1	0.1	0.1
*a*19:0	0.2	0.2	0.3	0.3
Subtotal[d]	2.3	2.4	2.8	3.0
Monounsaturated Fatty Acids				
16:1(n-12)	0.2	0.2	0.2	0.1
16:1(n-11)	0.1	0.1	0.1	tr[e]
16:1(n-10)	0.5	0.8	0.3	0.4
16:1(n-9)	0.1	0.2	0.1	0.1
16:1(n-7)	1.4	1.1	0.9	0.9
16:1(n-5)	0.3	0.3	0.2	0.3
17:1(n-8)	0.3	0.2	0.2	0.2
18:1(n-13)	0.8	0.9	0.4	0.2
18:1(n-9)	4.5	4.4	2.8	2.4
18:1(n-7)	3.4	3.4	2.8	2.8
18:1(n-5)	0.1	0.2	0.2	0.2
20:1(n-15)	0.1	0.1	tr	0.1
20:1(n-14)	0.1	0.3	0.2	0.2
20:1(n-13)	1.1	1.0	1.0	1.1
20:1(n-9)	0.6	0.8	1.3	1.2
20:1(n-7)	2.7	2.7	3.9	3.7
Subtotal[d]	15.6	15.8	14.0	13.2

Table 19 (Continued) Percentage composition of the fatty acids in adult *Crassostrea gigas*

Fatty acids	*"Control" a* Large[c]	Small	*Lyophilized b* Large	Small
Polyunsaturated Fatty Acids				
18:2(n–6)	2.0	2.0	1.3	1.0
18:3(n–6)	0.2	0.2	0.1	0.1
18:3(n–3)	2.1	2.1	1.5	1.1
18:4(n–3)	4.3	4.6	3.5	2.9
20:2(n–6)	0.3	0.3	0.3	0.3
20:3(n–6)	0.2	0.2	0.1	0.1
20:3(n–3)	0.2	0.2	0.1	0.1
20:4(n–6)	2.6	2.4	3.4	3.9
20:4(n–3)	0.7	0.8	0.7	0.6
20:5(n–3)	15.7	15.3	15.9	17.0
21:5(n–3)	0.8	0.7	0.6	0.6
22:4(n–6)	0.2	0.2	0.3	0.3
22:5(n–6)	0.5	0.5	0.6	0.6
22:5(n–3)	1.3	1.2	1.4	1.4
22:6(n–3)	20.7	20.2	24.3	25.0
Subtotal[d]	51.8	51.1	54.4	55.5
Non–Methylene Interrupted Fatty Acids				
20:2(5,11)	0.2	0.2	0.1	0.1
20:2(5,13)	0.6	0.7	0.3	0.3
22:2(7,13)	0.9	0.9	1.3	1.4
22:2(7,15)	2.6	2.5	3.4	3.7
22:3(7,13,16)	0.3	0.2	0.4	0.5
Subtotal	4.5	4.5	5.5	5.9
Not identified	0.6	0.7	1.3	1.0

[a] "Control", solvent extracted lipids of non–lyophilized sample; [b] Lyophilized, solvent extracted lipids of freeze dried sample; [c] large, mean soft tissue weight 8.9±0.5 g; small, mean soft tissue weight 5.8±0.4 g; [d] Subtotals include contributions from minor fatty acids 21:0, 23:0, *i*14:0, *a*15:0, br17:0, 22:1, 24:1, 16:2(n–4, 18:2(n–9?, 18:2(n–3); [e] tr, trace = <0.05%

Table 20. Percentage compositions and concentrations of major sterols in adult *Crassostrea gigas*.

Sterol	Trivial name	*"Control"* [a]		*Lyophilized* [b]	
		Large[c]	Small[c]	Large	Small
24–Norcholesta–5,22–dien–3β–ol	–	5.1	5.4	4.9	5.2
27–Nor–24–methylcholesta–5,22E–dien–3β–ol	Occelasterol	4.9	5.1	4.9	5.0
Cholesta–5,22E–en–3β–ol	*Trans*–22–dehydrocholesterol	12.2	12.7	11.6	12.3
5α–Cholest–22E–en–3β–ol	*Trans*–22–dehydrocholestanol	0.6	0.6	0.5	0.6
Cholest–5–en–3β–ol	Cholesterol	28.6	26.7	27.1	27.6
5α–Cholestan–3β–ol	Cholestanol	2.2	2.0	1.9	2.0
Cholesta–5,24–dien–3β–ol	Desmosterol	0.5	1.5	1.5	2.3
24–Methylcholesta–5,22E–dien–3β–ol	Brassicasterol/crinosterol	13.5	14.8	16.8	15.7
24–Methylcholesta–5,24(28)–dien–3β–ol	24 Methylenecholesterol	12.2	12.0	13.8	13.4
24–Methylcholest–5–en–3β–ol	Campesterol/dihydrobrassicasterol	1.7	1.7	1.8	1.8
24–Ethylcholesta–5,22E–dien–3β–ol	Stigmasterol/poriferasterol	1.9	1.3	2.1	2.0
24–Ethylcholest–5–en–3β–ol	Sitosterol/clionosterol	6.0	5.7	0.8	0.9
24–Ethylcholesta–5,24(28)E–dien–3β–ol	Fucosterol	1.1	1.2	1.3	1.1
24–Ethylcholesta–5,24(28)Z–dien–3β–ol	Isofucosterol	7.9	7.8	9.1	8.3
24–*n*–Propylcholesta–5,24(28)–dien–3β–ol	24–Propylidenecholesterol	1.5	1.4	1.9	1.7
Total sterols					
Concentration (mg/g wet weight)		1.2	1.2	1.1	0.9
Concentration (mg/g dry weight)		nd[d]	5.4	5.5	5.6

[a] "Control" i.e. solvent extracted lipids of non–lyophilized sample.
[b] Lyophilized i.e. solvent extracted lipids of freeze dried sample.
[c] large: mean soft tissue weight 8.9±0.5 g; small: mean soft tissue weight 5.8±0.4 g.
[d] nd – not determined.

A feeding experiment involving 3 species of cryptomonad and *Isochrysis* (T.iso) fed to oyster spat has been carried out and biochemical analyses are in progress. preliminary data show differences in growth rates. Further work on *Crassostrea* is scheduled to be carried out in conjunction with studies planned by Dr M. Brown (CSIRO Division of Fisheries) as part of FRDC grant 94/83 "Increased production of juvenile Pacific oysters (*Crassostrea gigas*) through supplementary feeding".

Another experiment was carried out in collaboration with Dr John Nell from the NSW Fisheries Brackish Water Fish Culture Research Station to test the use of marine yeasts and bacteria as alternative live foods, with lower production costs, to microalgae for the Sydney Rock Oyster *Saccostrea commercialis* larvae and spat. Fatty acid analyses were performed on 8 marine yeasts, 7 marine bacteria and one sample each of oyster spat and larvae. Dr Nell found that replacing 86% of the successful algal diet with a culture of bacteria (for larvae) or yeast (for spat) produced up to 67.2% or 81.4% of the growth on the normal algal feed. He concluded that there is commercial potential in the use of live yeast cultures as partial replacements for live algal diets in these oysters.

Our results show that although the major fatty acid in most of the yeasts is the polyunsaturated fatty acid, linoleic acid [18:2(n–6)], none of the yeasts or bacteria contained the long–chain omega–3 series of polyunsaturated fatty acids 20:5(n–3) and 22:6(n–3), both thought to be essential for marine species. Note that the oyster spat and larvae contained both fatty acids [20:5(n–3) as 4.4% and 7.1%, and 22:6(n–3) as 7.1% and 10.7% of the total fatty acids in spat and larvae respectively]. Due to this lack of 20:5(n–3) and 22:6(n–3), we would not recommend complete replacement of live algal feeds with presently available marine yeasts or bacteria (Brown *et al.*, 1995a). It should also be investigated at what point the oyster growth may become limited when fed on a substitute diet deficient in 20:5(n–3) or 22:6(n–3). There appears to be great potential for the replacement of live algal foods in aquaculture if similar yeast or bacteria containing 20:5(n–3) or 22:6(n–3) can be found. Further work in this direction is being undertaken by Dr Peter Nichols (CSIRO Division of Oceanography) as part of Antarctic CRC studies.

5.4 Feeding Experiments with Zooplankton (Objective 5)

During 1993 we placed an increasing emphasis on the transfer of lipids from the diet to the animal being reared through the use of controlled feeding experiments. Much of this work has been carried out in collaboration with staff and students from the University of Tasmania (Lecturers Dr Greg Maguire and Dr David Ritz and co–supervised students Greg Maloney, Piyapong Chotipunti and Anna Minchin).

Our previous studies showed that the polyunsaturated fatty acid content of rotifers fed on the prymnesiophyte alga *Pavlova lutheri* increased dramatically within just a few hours of feeding (Nichols *et al.*, 1989). In contrast, analyses carried out for Mahanga Bay Hatchery of *Artemia* fed on several PUFA–rich algae showed that these animals contained

much lower proportions of C_{22} PUFA. Our data suggest that *Artemia* is not a good source of 22:6(n–3) even when it is fed on microalgae containing high proportions of this essential fatty acid. However, discussions with Dr Patrick Lavens of the Aquaculture Laboratory in Belgium indicates that new strains with enhanced levels of 22:6(n–3) may soon be on the market.

As a part of a collaborative project with Dr Greg Maguire and students from the University of Tasmania Key Centre for Aquaculture Research we have compared the effect of three zooplankton diets having different lipid compositions on the compositions of megalopa larvae of Blue swimmer crab (a potential mariculture species) from Queensland. In a similar study a comparison was made of the effects of two zooplankton diets (*Artemia* and *Brachionus*) and an artificial diet (Frippak) each having a different lipid composition on the mysis larvae of the black tiger prawn *Penaeus monodon* (Figure 13). In both studies it was demonstrated that the crustaceans rapidly assimilated the dietary fatty acids resulting in fatty acid profiles similar to the foods with which they had been fed. However, whereas the fatty acid profiles of rotifers closely matched the composition of their feeds (whether microalgae or an artificial feed), the *Artemia* incorporated very little of the C_{20} and C_{22} PUFA present in the algae (Figure 13). Prawns reared on *Artemia* still had high levels of 18:3 present in the diet. Papers discussing these results are presently being prepared.

In a collaborative project with the Tasmanian Department of Sea Fisheries a comparison was made of the fatty acids in the flesh of wild caught and caged samples of the striped trumpeter which is a premier eating fish and also a candidate for marine farming. Such studies may help establish potential changes in the taste and palatability of the fish flesh due to differences in diet (in this case salmon feed pellets were used which contain high amounts of terrestrial plant type PUFA). Samples of fish larvae and various diets were also collected for analysis as part of feeding trials investigating the relationship between feed quality and growth rates of striped trumpeter. Unfortunately, due to an outbreak of *Aeromonas* the fish culture facility at the Department of Sea Fisheries was placed under quarantine, and samples already taken for research purposes had to be destroyed.

Although no fish larvae culture experiments could be carried out, several zooplankton feed samples were analysed. The fatty acid and total lipid composition of 10 samples of *Artemia salina* and 5 samples of *Brachionus plicatilus* fed different diets were analysed. Starved *Artemia* had 62–119 mg/g lipid whereas fed animals had 64–217 mg/g lipid. *Brachionus* lipid levels were also quite variable (87–147 mg/g), and lowest lipid content occurred in animals fed on Frippak. All diets boosted 20:5 and 22:6 levels in *Artemia* and *Brachionus* (little or no 22:6 was present in starved samples) with best results from Superselco. Analysis of wild zooplankton (mostly copepods) collected with the new Baleen harvester (Frish polyculture harvester) and of a cultured zooplankton (a daphnid) regularly used by the mariculture industry showed the former to contain significantly higher proportions of both of these essential PUFA. Work is in progress to study other natural zooplankton in collaboration with Dr John Purser and PhD student Andria Marshall.

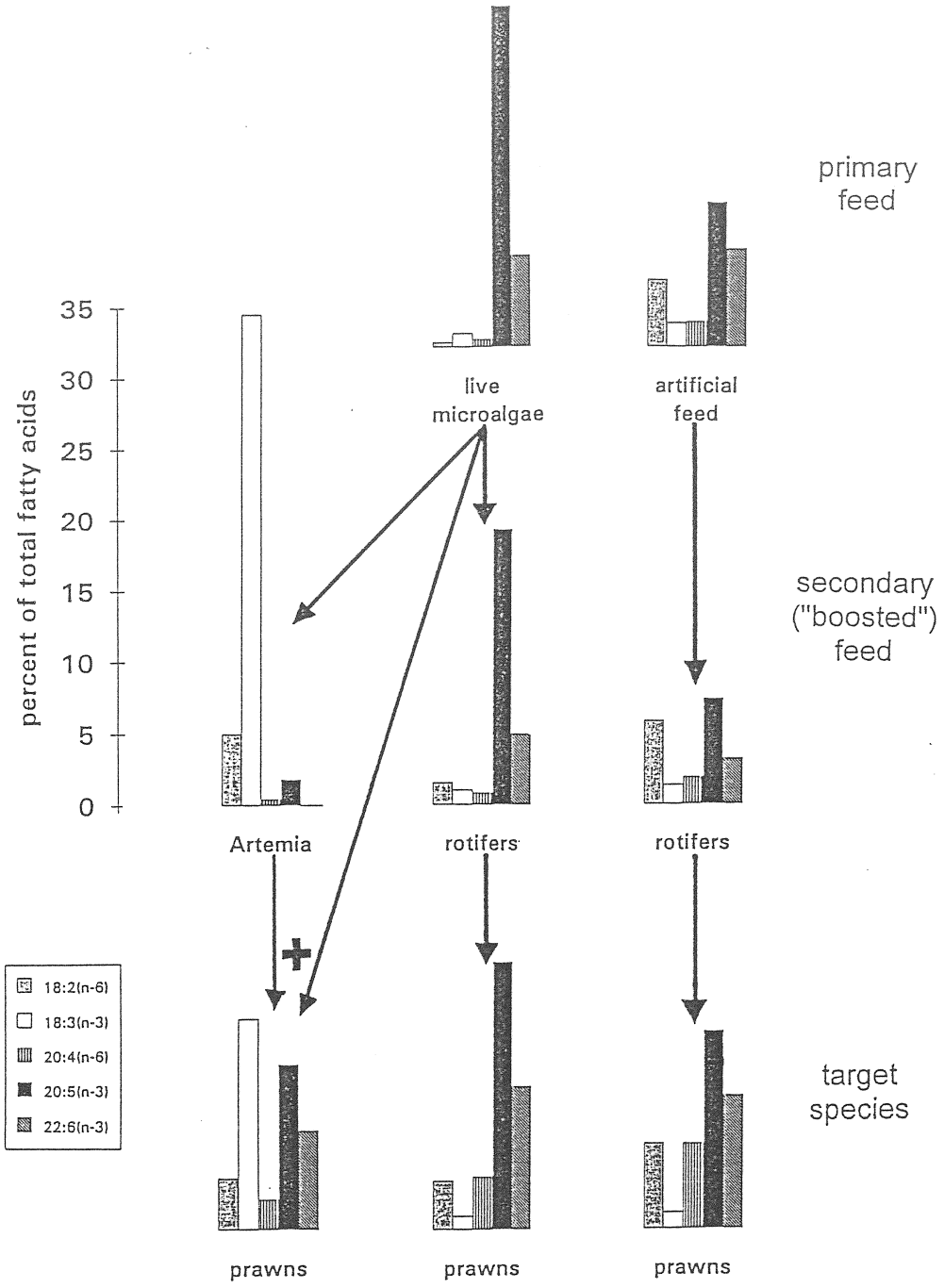


Figure 13. Distribution of major PUFA in prawn larvae fed rotifers and *Artemia* which had been boosted on an algal diet. Data for rotifers maintained on an artificial diet are also shown.

5.5 Communication activities and interactions with industry (Objective 6).

The number of our research collaborators increased steadily during the course of the project and we have been able to provide information to many aquaculture companies and research groups around Australia. Many of these have been in the form of reports or have resulted in jointly–authored papers (see publication list). Numerous phone enquiries were also answered. A large number of talks and posters were presented to scientific conferences and workshops both in Australia and overseas. These are listed at the end of this report. Many of these presentations were directed specifically at the mariculture industry and associated scientific groups. For example, a paper entitled "Nutritional properties of microalgae for mariculture" was presented by Dr M. Brown to the Sixth International Symposium on Fish Nutrition and Feeding" held in Hobart. This review paper incorporated fatty acid and lipid data produced from our FRDC–funded project as well as information obtained from other FRDC–funded projects (86/81, 88/69 and 90/63). A poster was also presented at the "Second International Symposium on Abalone Biology, Fisheries and Culture" held at the University of Tasmania, February 7–11, 1994. Some of our more unusual findings on algal lipids have also proven to be of interest to organic geochemists and biotechnologists which provided opportunities to present our data to a wider audience.

A limited study was carried out to determine the fatty acid composition of antarctic (spiny icefish – *Chaenodraco wilsoni*) and subantarctic fish (mackerel icefish – *Champsocephalus gunnari*, toothfishes – *Dissostichus eleginoides*) in comparison with temperate species such as the striped trumpeter (*Latris lineata*). The flesh of all species contained high concentrations of PUFA, and thus would be a good source of PUFA–rich fish oils for use in mariculture feeds. However, analysis of a commercially rendered oil produced from processing waste from *D. eleginoides* contained much lower concentrations of PUFA and a high content on monounsaturated fatty acids as has been observed for some other species. These results were published (Nichols *et al.*, 1994).

In a study with Dr. S. Percival of SALTAS, a comparison was made between two methods for the fatty acid determination of the steam pelleted feeds of Atlantic salmon and significant differences were noted between the method routinely used at the CSIRO Division of Oceanography, and the direct saponification method of Kovacs *et al.* (1979). There were significant differences between the amounts and compositions of the lipids extracted. These preliminary analyses established that the extraction method of Bligh and Dyer (1959) was superior to the direct saponification method of Kovacs *et al.* (1979) for the determination of lipids and fatty acids in pelleted fish food but not for fish faeces. In view of these findings, we recommend that the samples of fish food and faeces should be solvent extracted to determine the solvent extractable lipids and the remaining solids be directly saponified to determine the bound lipids. We also recommend that lipid determinations be quantified both gravimetrically and by Iatroscan TLC–FID. The data produced was highly reproducible so in future experiments it may only be necessary to make duplicate analyses.

Preliminary experiments were also carried out to examine the lipid requirements of abalone in conjunction with staff from Marine Shellfish Hatcheries, Bicheno, Tasmania. These studies indicated that particular polyunsaturated fatty acids are preferentially assimilated from the natural diet by blacklip abalone implying that they are essential nutrients. The fatty acid composition of the abalone flesh was found to change according to the composition of its diet when fed 2 artificial diets and a macroalga. A proposal for new funding to develop this aspect of the work entitled "Optimisation of essential lipids in artificial feeds for Australian abalone" by J. K. Volkman, G. A. Dunstan and G. B. Maguire has been funded by the FRDC.

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Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition. October 27–29, 1981. Lewes/Rehoboth Beach, Delaware.. Louisiana State University Division of Continuing Education, Baton Rouge Louisiana, pp. 272–291.

Whyte, J. N. C., Bourne, N. and Hodgson, C. A., 1989. Influence of algal diets on biochemical composition and energy reserves in *Patinopecten yessoensis* (Jay) larvae. *Aquaculture* 78: 333–47.

Witt, U., Quantz, G., Kuhlmann, D. and Kattner, G., 1984. Survival and growth of turbot larvae *Scophthalmus maximus* L. reared on different food organisms with special regard to long–chain polyunsaturated fatty acids. *Aquacult. Eng.*, 3: 177–190.

7. Outcomes compare with objectives

Substantial progress was made in addressing all of the objectives of the project. The experiments for characterization of algal lipid compositions (Objectives 1–3) were completed and most of the data have been published in the scientific literature (see Section 9). Some further papers are still being prepared. Feeding experiments (Objectives 4 and 5) have produced some very interesting data, but progress was limited due to technical difficulties of rearing the animals. The proposed research on *Crassostrea* was postponed to coincide with research being carried out by M. Brown as part of FRDC grant 94/83, and initial work on abalone nutrition formed the basis for a new FRDC grant 94/085 entitled "Optimisation of essential lipids in artificial feeds for Australian abalone" by J. K. Volkman, G. A. Dunstan and G. B. Maguire. Many communication activities (Objective 6) were undertaken, including presentations at conferences and workshops, numerous discussions with hatcheries and scientific groups, preparation of analytical reports and publication of scientific papers. Details of these activities are provided in Section 9.

8. Benefits

In less than a decade the mariculture industry in Australia has increased dramatically in value, diversity and sophistication. This increase has been underpinned by research at al levels aimed at both understanding basic processes and applied needs of the industry. Microalgae are still an essential feature of most mariculture operations, particularly to meet the nutritional needs of early life stages of most marine animals, despite attempts to find alternative feeds. These algae need to be carefully chosen to meet the nutritional requirements of the animal being reared while at the same time must be robust and easy to culture.

Our research in combination with that undertaken as part of FRDC grants and 90/63 to colleagues Jeffrey, Garland and Brown has provided a comprehensive database on the nutritional qualities of microalgae (lipids, proteins, carbohydrates, vitamins) used as live feedstocks by the industry. Hatcheries can now order species from the CSIRO microalgal supply service operated by the Division of Fisheries that meet their needs and are delivered in good condition in 3 days or less. This service has been so successful that almost all species used by the industry are now provided by CSIRO. Over 2600 starter cultures have been provided in the past 7 years representing a direct import replacement benefit estimated at $255000 (Jeffrey *et al.*, 1994).

A major benefit of the work has been the increased knowledge of the nutritional qualities of microalgae and a better understanding by the industry of the need for adequate levels of polyunsaturated fatty acids in the diet of marine animals. Expert advice has been communicated to many different groups around Australia, often by phone or personal contact but also through presentations at workshops and conferences. Hatcheries have sought our advice as to whether a particular species might be suitable for their needs and we have been able to make recommendations based on our biochemical data and other information. An analytical capability for lipid and fatty acid analysis has been established which we believe is of international standard. Analytical data have been provided to numerous research groups and companies (see reports section) as an aid to their research work. Advice and training has also been provided to other research groups and students which has contributed to the development of these analytical techniques elsewhere in Australia.

Detailed fatty acid compositional data have been determined for 56 microalgal species, and most of this information has been published. Characteristic features of each of the major algal classes, and distinctive features of some of the commonly used genera, have been determined. This has provided a baseline for comparison of results obtained from feeding trials and pointed to related algal species which should prove to be useful feeds. These data combined with improved knowledge of the environmental tolerances of different species has allowed us and other groups to identify new species better suited for particular applications; some of these are now being investigated in feeding trials (*Navicula* sp., *Nitzschia closterium*, *Nannochloropsis* sp. and *Pavlova* sp.).

Misunderstandings about the taxonomy of "marine Chlorella" have been resolved by confirming Japanese work that this alga is a eustigmatophyte and not a chlorophyte. It is now clear from our work that most species from the Chlorophyceae are in fact poor sources of essential PUFA. On the other hand, the eustigmatophytes have been shown to be excellent feeds for mariculture and excellent sources of PUFA.

Important new data were obtained on the effect of culture conditions on the lipid composition of microalgae grown in large–scale batch culture. This work has confirmed that laboratory–scale culture provide a good general insight into lipid compositions of microalgae, but also that these compositions can be manipulated further to optimise the content of desired biochemicals by judicious choice of culturing conditions at the hatchery.

Our work on boosting fatty acid composition in zooplankton species has revealed that there is considerable variability in the results obtained with commercial boosters and with different species of microalgae. This is of practical value to hatchery staff since it is the larval and juvenile stages of many marine animals where nutritional requirements are most critical.

Studies of fatty acid compositions of marine animals from the wild and natural feeds have provided baseline data to compare with results obtained from mariculture. These data can also be used as a likely guide to the specific requirements of the animal which can be used when defining the chemical compositions of artificial feeds. Some of our findings for abalone suggest that these animals have an unusual fatty acid composition and feeding trails are in progress to determine their specific requirements for essential fatty acids.

9. Intellectual property

This project generated a significant amount of intellectual property in the form of:

- new information regarding the lipid and fatty acid content of microalgae used by the Australian aquaculture industry,
- identification of new species which might be more suitable feeds,
- development of analytical methods based on TLC–FID for the identification of lipid classes in small samples of marine algae, animals and feeds,
- further refinement of methods for identifying fatty acids in complex mixtures,
- improvements in lipid extraction from freeze–dried samples
- discovery of some of the factors influencing biochemical composition in microalgae in batch culture,
- knowledge of rates of fatty acid uptake by zooplankton fed microalgae and booster feeds,
- development of a comprehensive database of fatty acid, lipid and sterols compositions for 56 species of microalgae,

- formulation of general statements about typical fatty acid and lipid compositions expected in different classes of microalgae.
- knowledge of the biochemical composition of marine animals including zooplankton, oysters, rock lobster, prawns and abalone which may be indicative of nutritional requirements of these animals.

In view of the formative state of the industry, we have sought to distribute this information as widely as possible and so no patents have been taken out. Much of the fatty acid data obtained were distributed directly to the aquaculture industry as articles written by us for inclusion in the regular newsletter "Microalgae for Mariculture" (7 issues). This was compiled by Drs Jeffrey and Garland and funded by FIRTA Grant 86/81 for distribution to over 300 individuals and companies involved with the Australian mariculture industry.

In additional to personal contacts with industry personnel, our results were also communicated to mariculturists who attended the various "Microalgae for Mariculture" workshops held at the University of Tasmania. Talks on nutrition were given by Drs Volkman, Brown and Nichols while other aspects of microalgal culture were extensively covered in lectures by Drs Jeffrey, Garland, Rees and Blackburn. Attendees were also given guided tours of facilities at the CSIRO Marine Laboratories during which many informal discussions with industry personnel were held.

Numerous presentations were given at conferences and workshops, reports to FRDC, industry and other research groups plus a large number of scientific papers. Details of these are provided in the following lists.

9.1 Scientific papers

(in date order)

Bremner H. A., Volkman J. K., Krasnicki T. and Gibson R. (1989) The good oil: nutritionally important oils in Tasmanian fish. *Aust. Fisheries* **48(5)**, 28–29.

Volkman J. K., Jeffrey S. W., Rogers G. I., Nichols P. D. and Garland C. D. (1989) Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *J. Exp. Mar. Biol. Ecol.* **128**, 219–240.

Volkman J. K. (1989) Fatty acids of microalgae used as feedstocks in aquaculture. In *Fats for the Future II.* (ed R. C. Cambie), pp. 263–283, Ellis Horwood, Chichester.

Nichols N., Skerratt J., Elliott N. and Volkman J. (1989) A simple procedure to fully identify monounsaturated alkyl chains in marine wax esters. In *Food Forums*

Proceedings, Tenth Australian Symposium on Analytical Chemistry, Brisbane, August 1989 pp. 185–190.

Nichols P. D., Holdsworth D. G., Volkman J. K., Daintith M. and Allanson S. (1989) High incorporation of essential fatty acids by the rotifer *Brachionus plicatilis* fed on the prymnesiophyte alga *Pavlova lutheri*. *Aust. J. Mar. Freshw. Res.* **40**, 645–655.

Deprez P. P., Volkman J. K. and Davenport S. R. (1990) Squalene content and neutral lipid composition of livers from deep–sea sharks caught in Tasmanian waters. *Aust. J. Mar. Freshw. Res.* **41**, 375–387.

Volkman J. K., Kearney P. and Jeffrey S. W. (1990) A new source of 4–methyl sterols and 5α(H)–stanols in sediments: prymnesiophyte microalgae of the genus *Pavlova*. *Organic Geochemistry* **15**, 489–497.

Volkman J. K. and Nichols P. D. (1991) Applications of thin layer chromatography–flame ionization detection to the analysis of lipids and pollutants in marine and environmental samples. *J. Planar Chromatog.* **4**, 19–26.

Volkman J. K., Dunstan G. A., Jeffrey S. W. and Kearney P. S. (1991) Fatty acids from microalgae of the genus *Pavlova*. *Phytochemistry* **30**, 1855–1859.

Hallegraeff G. M., Nichols P. D., Volkman J. K., Blackburn S. I. and Everitt D. A. (1991) Pigments, fatty acids, and sterols of the toxic dinoflagellate *Gymnodinium catenatum*. *J. Phycol.* **27**, 591–599.

Volkman J. K., Barrett S. M., Dunstan G. A. and Jeffrey S.W. (1992) C_{30}–C_{32} alkyl diols and unsaturated alcohols in microalgae of the class Eustigmatophyceae. *Organic Geochemistry* **18**, 131–138.

Dunstan G. A., Volkman J. K., Jeffrey, S. W. and Barrett S. M. (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae 2. Lipid classes and fatty acids. *J. Exp. Mar. Biol. Ecol.* **161**, 115–134.

Volkman J. K., Dunstan G. A., Barrett S. M., Nichols P. D. and Jeffrey S. W. (1992) Essential polyunsaturated fatty acids of microalgae used as feedstocks in aquaculture. In *Proceedings Aquaculture Nutrition Workshop*, Salamander Bay, 15–17 April 1991. (eds G. L. Allan and W. Dall), pp. 180–186, NSW Fisheries, Brackish Water Fish Culture Research Station, Salamander Bay.

Volkman J. K., Brown, M. R., Dunstan G. A. and Jeffrey S. W. (1993) Biochemical composition of marine microalgae from the class Eustigmatophyceae. *J. Phycol.* 29, 69–78.

Volkman J. K., Barrett S. M., Dunstan G. A. and Jeffrey S. W. (1993). Geochemical significance of the occurrence of dinosterol and other 4–methyl sterols in a marine diatom. *Organic Geochemistry* **20**, 7–15.

Dunstan G. A., Volkman J. K., Barrett S. M. and Garland C. D. (1993). Changes in the lipid composition and maximisation of the polyunsaturated fatty acid content of three microalgae grown in mass culture. *J. Appl. Phycol.* **5**, 71–83.

Nichols P. D., Nichols D. S. and Volkman J. K. (1993) Recent developments with marine oil products in Australia. *Chem. Aust.* **60(7)**, 336–340.

Brown M. R., Dunstan G. A., Jeffrey S. W., Volkman J. K., Barrett S. M. and LeRoi J.–M. (1993) The influence of irradiance on the biochemical composition of the prymnesiophyte *Isochrysis* sp. (clone T–ISO). *J. Phycol.* **29**, 601–612.

Dunstan G. A., Volkman J. K. and Barrett S. M. (1993). The effect of lyophilization on the solvent extraction of lipid classes, fatty acids and sterols from the oyster *Crassostrea gigas*. *Lipids* **28**, 937–944.

Dunstan G. A., Volkman J. K., Barrett S. M., LeRoi J.–M. and Jeffrey S. W. (1994). Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). *Phytochemistry* **35**, 155–161.

Nichols D. S., Williams R., Dunstan G. A., Nichols P. D. and Volkman J. K. (1994) Fatty acid composition of Antarctic and temperate fish of commercial interest. *Comp. Biochem. Physiol.* 107B, 357––363.

Volkman J. K., Barrett S. M. and Dunstan G. A. (1994). C_{25} and C_{30} highly branched isoprenoid alkenes in laboratory cultures of two marine diatoms. *Organic Geochemistry* **21**, 407–413.

Volkman J. K., Barrett S. M., Dunstan G. A. and Jeffrey S. W. (1994). Sterol biomarkers for microalgae from the green algal class Prasinophyceae. *Organic Geochemistry* **21**, 1211–1218.

Jeffrey S. W., Brown M. R. and Volkman J. K. (1994). Haptophytes as feedstocks in mariculture. In. *The Haptophyte Algae* (eds J. C. Green and B. S. C. Leadbeater), pp. 287–302. Clarendon Press, Oxford.

Conte M. H., Volkman J. K. and Eglinton G. (1994). Lipid biomarkers of the Haptophyta. In. *The Haptophyte Algae* (eds J. C. Green and B. S. C. Leadbeater), pp. 351–377. Clarendon Press, Oxford.

Barrett S. M., Volkman J. K., Dunstan G. A. and LeRoi J.–M. (1995) Sterols of 14 species of marine diatoms (Bacillariophyta). *J. Phycol.* (in press).

Brown M. R., Jeffrey S. W., Volkman J. K. and Dunstan G. A. (1995) Nutritional properties of microalgae for mariculture. *Proceedings of the VI International Symposium on Fish Nutrition and Feeding*. (in press).

Brown, M. R., Barrett, S. M., Volkman, J. K., Nearhos, S. P., Nell, J. A. and Allan, G. L. (1995). Biochemical composition of new yeast and bacteria evaluated as food for bivalve aquaculture. *Aquaculture* (in prep.).

Brown, M. R., Dunstan, G. A., Norwood, S. A. and Miller, K. A. (1995). The effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*. *J. Phycol.* (in prep.).

Note: Publications from 1989–1992 were generated from related research initially undertaken as part of FIRTA 88/69.

9.2 Reports to Industry and Research Agencies

Volkman J.K. (1992) GC–MS analysis of sterols in two yeast samples from CUB Ltd. Report 92–CUB1 for Carlton and United Breweries, Melbourne.

Nichols D.S., Nichols P.D. and Volkman J.K. (1992) Lipid class and fatty acid composition of AGAL oils A and B. Report 92–AGA–1 to Australian Government Analytical Laboratories, Tasmania.

Nichols D.S., Nichols P.D. and Volkman J.K. (1992) Lipid class and fatty acid composition of Jack Mackerel oil. Report 92–IND–1 to Industrial Fish Pty. Ltd., Triabunna.

Nichols D.S., Nichols P.D. and Volkman J.K. (1992) Lipid class and fatty acid composition of Jack Mackerel oil. Report 92–IND–2 to Industrial Fish Pty. Ltd., Triabunna.

Nichols D.S., Nichols P.D. and Volkman J.K. (1992) Oil, proximate and fatty acid composition of freshwater copepods and daphnids obtained using the Frish polyculture harvester. Report 92–FRISH–2 to Frish Pty. Ltd., Tasmania.

Nichols D.S and Volkman J.K. (1993) Fatty acid composition of Jack Mackerel oil. Report 93–ALL–1 to Allison Laboratories, Hobart.

Nichols D.S., Nichols P.D. and Volkman J.K. (1993) Oil, proximate and fatty acid composition of copepods and daphnids obtained from Werribee Treatment Complex using the Frish polyculture harvester. Report 93–ZOOTECH–1 to Zootech Industrial Polyculture Pty. Ltd., Tasmania.

Dunstan G. A., Volkman J. K., Nichols P. D. and Bakes M. J. (1993) Total lipid and fatty acid composition of steam pelleted fish feed. Report 93–Salt4 to SALTAS.

Barrett S. M., Dunstan G. A. and Volkman J. K. (1994) Fatty acid composition of *Artemia salina* and *Brachionus plicatilus* fed on artificial diets. Report 94–SF1 for Tasmanian Department of Primary Industry and Fisheries, Hobart.

9.3 Conference Presentations

J. K. Volkman*, S. M. Barrett, G. A. Dunstan and S. W. Jeffrey (1992). *Advances in the biomarker chemistry of marine microalgae*. Talk at the Third Conference on Petroleum Geochemistry and Exploration in the Afro–Asian Region, Melbourne, Australia, February 10–13.

S. W. Jeffrey*, M. R. Brown and J. K. Volkman (1992) *Microalgae in Australian mariculture*. Talk at Marine Biotechnology Symposium, 1992. Shimizu, Japan, February.

J. K. Volkman*, G. A. Dunstan, S. M. Barrett and S. W. Jeffrey (1992) *Marine microalgae as a source of essential polyunsaturated fatty acids*. Talk at Third International Congress on Essential Fatty Acids and Eicosanoids. Adelaide, South Australia, March 1–5.

J. K. Volkman, S. M. Barrett, G. A. Dunstan and S. W. Jeffrey (1992) *Biomarkers for marine microalgae*. Poster at Gordon Research Conference on Organic Geochemistry, Holderness School, New Hampshire, U.S.A., August 10–14.

P. D. Nichols*, D. S. Nichols, G. A. Dunstan and J. K. Volkman (1992). *Biochemical aspects of mariculture nutrition.* Industrial Polyculture Meeting, Werribee Treatment Complex, Victoria, October 26.

M. R. Brown*, S. W. Jeffrey and J. K. Volkman (1992) *The nutritional properties of microalgae used in Australian mariculture*. 10th Australasian Society for Phycology and Aquatic Botany Conference, Sydney, December 6–9.

M. R. Brown*, S. J. Norwood, K. A. Miller and G. A. Dunstan (1992) *Effects of light intensity and photoperiod on the biochemical composition of Thalassiosira pseudonana.* Poster, 10th Australasian Society for Phycology and Aquatic Botany Conference, Sydney, December 6–9.

S. W. Jeffrey, M. R. Brown and J. K. Volkman* (1993) *Prymnesiophytes as feedstocks in mariculture.* Talk at "The Biology of the Prymnesiophyta", Plymouth, UK, March 29–April 1.

J. K. Volkman*, S. M. Barrett, G. A. Dunstan and S. W. Jeffrey (1993). *Novel hydrocarbons, sterols and alkyl diols from marine microalgae*. Talk at the 84th American Oil Chemists' Society Annual Meeting, Anaheim, California, April 25–28.

G. A. Dunstan, J. K. Volkman* and S. M. Barrett (1993). *The effect of lyophilization on the solvent extraction of lipids from the oyster Crassostrea gigas (Thunberg)*. Talk at the 84th American Oil Chemists' Society Annual Meeting, Anaheim, California, April 25–28. (Winner of award for outstanding paper presentation)

P. D. Nichols*, J. K. Volkman and D. S. Nichols (1993). *Progress in the development of marine oil products in Australia*. Talk at the 84th American Oil Chemists' Society Annual Meeting, Anaheim, California, April 25–28. (Winner of award for outstanding paper presentation)

P. D. Nichols*, D. S. Nichols, M. Bakes and J. K. Volkman (1993) *Progress in the development of marine oil products in Australia.* Talk, AMSA Conference, Melbourne, July.

J. K. Volkman*, S. M. Barrett, G. A. Dunstan, S. I. Blackburn and S. W. Jeffrey (1993) *Biomarkers for marine microalgae.* Talk at the 16th International Meeting on Organic Geochemistry, Stavanger, Norway. September 20–24.

S. W. Jeffrey*, M. R. Brown and J. K. Volkman (1993) *The nutritional value of microalgae used in mariculture.* Talk, Fifteenth International Congress of Nutrition. Adelaide, September 26–October 1.

M. R. Brown*, S. W. Jeffrey and J. K. Volkman (1993) *Nutritional properties of microalgae for mariculture.* Talk, Sixth International Symposium on Fish Nutrition and Feeding. Hobart, Tasmania, October 4–7.

G. A. Dunstan, M. R. Brown, S. M. Barrett, J.–M. Leroi, S. W. Jeffrey and J. K. Volkman (1994). *Biochemical compositions of benthic diatoms used in juvenile abalone culture.* Poster at Second International Symposium on Abalone Biology, Fisheries and Culture, University of Tasmania, February 7–11.

* denotes speaker

10. Further Developments

The project fulfilled its main objective to determine the fatty acid and lipid composition of microalgae used by the Australian mariculture industry. We also identified new species having appropriate biochemical composition which might be more suitable species for use as feeds. There is now a need to trial these species under controlled conditions approximating those used in commercial operations to determine whether these species can replace existing feedstocks. Some work in this direction is being carried out by Dr Malcolm Brown and colleagues in the CSIRO Division of Fisheries with regard to oyster nutrition. There is also a continuing need to find strains better suited to tropical conditions. Some of the factors (light, growth stage, type of culturing) which can influence the biochemical composition of microalgae in large-scale batch culture have been studied. It would be valuable if these concepts were adopted in commercial hatcheries to optimise feed quality.

A significant limitation in the use of microalgae as live feeds is the expense and time needed to maintain and culture the algae. Alternative large-scale culture techniques such as continuous culture in chemostats should be investigated as alternatives to the batch culture techniques used presently in hatcheries. Some research on this topic is planned for the CSIRO Aquaculture initiative project on "Algal biotechnology". Another area which needs to be pursued is the production of algal pastes which have sufficient shelf life to obviate the need for algal culture at hatcheries. The extensive data base of biochemical information on microalgae that has now been acquired allows species with suitable nutritional profiles to be chosen for further investigation. This work is being undertaken by Dr Brown and colleagues from the CSIRO Division of Fisheries and by Dr M. Borowitzka from Murdoch University and colleagues.

Our understanding of the specific nutritional requirements of marine animals is still rudimentary for many species. Many diets have been formulated by trial and error and may be more expensive than necessary due to the inclusion of excessive amounts of particular nutrients. Feeding experiments with carefully defined diets are needed to determine minimum requirements for ingredients such as specific PUFA, sterols, vitamins, minerals etc. Some relevant studies are now underway as part of the Aquaculture CRC. A new FRDC-funded study by Volkman, Dunstan and Maguire of the nutritional requirements of abalone has commenced although this is limited to artificial feeds. More information is needed of the requirements of early growth stages of abalone when reared on diatom turfs.

Alternative live feeds should also be investigated, although work to date on oyster nutrition carried out with Dr John Nell for the Aquaculture CRC suggests that presently available yeasts and bacteria do not provide as good rates of growth and survival as microalgae presently used by the industry. However, this area shows promise particularly for those animals where requirements for essential long-chain polyunsaturated fatty acids are less critical and where mixed feeds are likely to be effective.

11. Budget

FRDC Funds:

	1991–92	1992–93	1993–94	1994–95
	$	$	$	$
Salaries & Wages	30474	64755	68999	35718
Operating Expenses	4700	10300	10500	5200
Travel Expenses	1300	2400	2600	1600
Capital Items	Nil	Nil	Nil	Nil
TOTAL	**36474**	**77455**	**82099**	**42518**

Total FRDC contribution: $238546

Total contribution from applicants: $214500

FINANCIAL CONTRIBUTION FROM APPLICANTS:

Salaries of chief investigators	$82500
Overhead, travel and operating support	$12000
10% of capital items used on project	$120000

12. Staff

Staff	Qualifications	Time on Project
Dr J. K. Volkman	B.Sc.(Hons) Ph.D.	15%
Dr P. D. Nichols	B.Sc.(Hons) Ph.D.	10%
Dr S. W. Jeffrey	B.Sc. M.Sc. Ph.D.	10%
Mr G. A. Dunstan	B.Sc.	100%
Ms S. M. Barrett	B.Sc.(Hons)	40%

Dr Volkman provided overall scientific direction and supervision for the project and prepared reports with assistance of Drs Nichols and Jeffrey. Fatty acid analyses were carried out by Mr Dunstan and sterol analyses were carried out by Ms Barrett. Dr Nichols assisted with more specialised chemical analyses, and Dr Jeffrey and staff were responsible for all aspects of microalgal culture. Publications and presentations were undertaken by all members of the team.

13. Acknowledgements

This research has substantially benefited from the contributions and discussions with many colleagues and industry associates. Daniel Holdsworth is thanked for technical assistance and for keeping our analytical facility operating at a high degree of efficiency. We thank Jeannie–Marie Leroi and Dr Sue Blackburn for their considerable assistance with algal cultures. Suzanne Norwood, Kelly Miller, Christine Farmer and Dr Tony Rees assisted with particular aspects of the algal work. Malcolm Brown, Christian Garland, Greg Maguire, Steve Percival, David Ritz, Lance Searl, John Nell and many colleagues provided valuable and helpful discussions.

The research described in this report was funded in part by the CSIRO Division of Oceanography, CSIRO Division of Fisheries, Fishing Industry Research Trust Account grant 86/81 to Jeffrey and Garland, Fishing Industry Research and Development Council grant 88/69 to Volkman, Nichols, Jeffrey and Garland, Australian Research Council grant A18831836 to Volkman and Jeffrey and Fisheries Research and Development Corporation grant 91/59 to Volkman, Nichols and Jeffrey.