FATTY ACID BIOMARKERS IN A COLD WATER MARINE ENVIRONMENT

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by

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ABSTRACT

Fatty acids are of great interest in a variety of disciplines, including oceanography, geochemistry, food science and biochemistry, and this has led to the development of diverse methods for their determination. This study was undertaken to establish optimal methods for fatty acid extraction and analysis and to apply those methods to samples in the marine environment. Several methods of lipid extraction, lipid fractionation, fatty acid methyl ester (FAME) formation and picolinyl ester synthesis were examined. For most sample types, a biphasic extraction mixture of 8:4:3 $CHCl_3$:MeOH:H_2O, followed by fractionation on silica gel and FAME formation with BF_3 gave optimal recoveries. Picolinyl derivatives of fatty acids are useful in structure determination with mass spectrometry and a new transesterification method for their synthesis was developed. In addition, the treatment of samples with high lipase activities with boiling water was effective in deactivating those enzymes and resulted in lower levels of free fatty acids, a breakdown product.

Combinations of all these methods were applied to biogeochemical and aquaculture projects. In the two very different environments of Trinity Bay and Barred Island Cove, the fatty acid composition of plankton and sediment trap samples was characterized by high levels of polyunsaturated fatty acids (20-50% of total fatty acids), indicating a substantial marine phytoplankton source, particularly diatoms. However, much higher levels of terrestrial plant and bacterial indicators in Barred Island Cove as compared to Trinity Bay illustrated the differences in the two environments. The fatty acid composition of blue mussels from Barred

Island Cove were also compared to that of natural phytoplankton populations. In terms of fatty acid nutritional needs, the phytoplankton seemed to be providing fatty acids in proportions closely approximating the bivalve's requirements. Another aquaculture interest is in establishing fatty acid biomarkers that may be used to indicate the presence of toxic algae. To this end, the fatty acid composition of the toxic diatoms *Pseudo-nitzschia multiseries* and *P. pungens* was determined. High levels of 16:4n-1 (>7%) were found in both species and that fatty acid may have potential in differentiating those *Pseudo-nitzschia* species from other diatoms. Thus, accurate analysis of fatty acids in cold water marine samples can provide insights into biogeochemical processes, food web connections and the chemotaxonomy of toxic phytoplankton.

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LIST OF ABBREVIATIONS

ai-	*anteiso-*
AMPL	Acetone-Mobile Polar Lipids
ALC	Aliphatic Alcohols
AOCS	American Oil Chemists' Society
DAG	Diacylglycerols
DGDG	Digalactosyl Diacylglycerols
DMA	Dimethyl Acetals
FAME	Fatty Acid Methyl Esters
FFA	Free Fatty Acids
GC	Gas Chromatography
GC-C-IRMS	Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry
GC-FID	Gas Chromatography-Flame Ionization Detection
GC-MS	Gas Chromatography-Mass Spectrometry
GE	Acylated Glyceryl Ethers
HC	Aliphatic Hydrocarbons
i-	*iso-*
MAG	Monoacylglycerols
ME	Methyl Esters
MGDG	Monogalactosyl Diacylglyerols
NMID	Non-Methylene Interrupted Dienes

PC	Phosphatidylcholine
PL	Phospholipids
PSP	Paralytic Shellfish Poison
PUFA	Polyunsaturated Fatty Acids
SE	Steryl Esters
SQDG	Sulphoquinovosyl Diacylglycerols
ST	Sterols
TAG	Triacylglycerols
TIC	Total Ion Chromatograph
TLC	Thin Layer Chromatography
TLC-FID	Thin Layer Chromatography-Flame Ionization Detection
WE	Wax Esters

Chapter 1 - GENERAL INTRODUCTION

Determination of fatty acids in marine samples is of great interest in a variety of fields, ranging from aquaculture to organic geochemistry. In aquaculture, the interest is mainly in fatty acid nutrition of fish and, in the most recent issues of Aquaculture and the Journal of World Aquaculture, approximately 25% of the articles involved fatty acid analysis. Fatty acids are also useful as biomarkers or signature compounds of organisms. In biogeochemical studies, this allows the determination of sources and sinks of organic material and contributes to knowledge of carbon cycling in the marine environment (De Baar *et al*, 1983; Conte *et al*, 1995; Harvey and Johnston, 1995; Wakeham *et al*, 1997a; Wakeham *et al*, 1997b). As markers of individual organisms, fatty acid signature compounds may also be useful in indicating the presence of toxic algae (Parrish *et al*, 1991). Accurate quantitation of these fatty acids is a challenge, particularly with samples from cold water environments which contain elevated levels of labile polyunsaturated fatty acids (PUFA). Errors may be introduced in the analysis during any one of a number of steps, including lipid extraction, fractionation and derivatization, and, given the proliferation of fatty acid analyses in diverse fields, a critical evaluation of existing methodology is timely. Furthermore, without some knowledge of the accuracy of analyses, comparisons of results from different groups may not be valid.

This study will attempt to remedy this situation by critically evaluating commonly employed techniques in fatty acid analysis. Numerous reviews exist (Schmitz and Klein, 1986; Christie, 1989; Hamilton *et al*, 1992; Christie, 1993; Dobson *et al*, 1995) describing a variety

of methods for quantitatively and qualitatively determining fatty acids, including extraction, fractionation, methylation and formation of derivatives for mass spectrometry. This thesis, however, will describe an improved method for accurate determination of fatty acids in cold water marine samples. Since optimization of instrumental parameters for both thin-layer chromatography-flame ionization detection of lipid classes (Parrish and Ackman, 1983a; Parrish and Ackman, 1983b) and gas chromatography of fatty acids (Albertyn *et al*, 1982; Ackman, 1986; Christie, 1989; Craske, 1993) have been extensively investigated in the past, it will not be further examined in this study but appropriate parameters and corrections will be employed as recommended in those studies.

Applications of fatty acids in environmental studies as determined by these optimal methods will also be demonstrated. The use of fatty acid biomarkers in biogeochemical studies and as markers of specific organisms is illustrated in Chapters 4, 5 and 6. The action of lipolytic enzymes is a particular problem in the determination of lipid class and fatty acid composition in diatoms and the effects of deactivation of these enzymes will be examined in Chapter 5. In addition, in Chapter 6, the fatty acid nutritional requirements of bivalves are discussed and compared to natural phytoplankton populations.

Chapter 2- SURVEY OF FATTY ACID COMPOSITION AND ANALYSIS OF MARINE SAMPLES

2.1 Introduction

Currently, there is much interest in the analysis of lipids in the marine environment. In living organisms, lipids have several functions which can be described as structural, storage or regulatory. Lipids associated with membranes have structural roles, while lipids used as sources of energy function as long-term fuel stores. Individual lipid molecules may even behave as chemical messengers and play a role in metabolism. These different functions require a variety of structures, and this in turn, makes lipids particularly suited as biomarkers. Biomarkers are molecules, structurally related to a specific source (Hedges and Prahl, 1993), which can be analyzed directly from the environment and can be used to determine *in situ* biomass both qualitatively and quantitatively (Sargent *et al*, 1987). Biomarkers may signal the presence of a particular organism and aid in establishing the distribution and abundance of that organism. They are useful in determining sources and fates of organic material in the marine environment (Wakeham and Beier, 1991). Lipid biomarkers may also be useful as a sort of early warning for the presence of toxic algae (Parrish *et al*, 1991).

Lipids are defined as substances that are insoluble in water and soluble in non-polar solvents such as chloroform and ethanol (Gurr and Harwood, 1991). Structures of these lipids vary widely and, to form a classification scheme, lipids have been divided into 16 classes based on structure (Parrish, 1988). These classes include, in order of increasing polarity,

aliphatic hydrocarbons (HC), polycyclic aromatic hydrocarbons, wax esters (WE), steryl esters (SE), short-chain esters, acylated glyceryl ethers (GE), triacylglycerols (TAG), free fatty acids (FFA), phthalate esters, free aliphatic alcohols (ALC), sterols (ST), diacylglycerols (DAG), monoacylglycerols (MAG), glycolipids, pigments and phospholipids (PL) (Figure 2.1). MAG, glycolipids and pigments are usually not determined individually but rather as one group, known as the acetone-mobile polar lipids (AMPL). In general, the term "polar lipid" includes only AMPL and PL. All other less polar lipid classes are referred to as neutral lipids. These neutral lipids, particularly TAG and WE, are commonly associated with long-term storage products, while the polar lipids play a structural role.

Lipids containing one or more esterified fatty acids are known as acyl lipids and these esters may be cleaved to yield the fatty acids. These fatty acids also have a variety of structures but, in marine organisms, fatty acids commonly contain from 14 to 22 carbon atoms of varying degrees of unsaturation. Several different types of nomenclature are applied to the fatty acids of acyl lipids. Most fatty acids were first isolated from a particular source and trivial names derived from the sources were often used. A systematic nomenclature recommended by the International Union of Pure and Applied Chemistry is also currently employed (Gurr and Harwood, 1991). This results in simultaneous use of systematic and trivial names. For example, *n*-hexadecanoic acid and palmitic acid are used interchangeably. A less ambiguous shorthand notation, based on the numbers of carbon atoms and double bonds, is also common. It takes the form of A:Bn-C, where A refers to the total number of carbon atoms, B to the number of ethylenic bonds and C to the number of carbon atoms from,

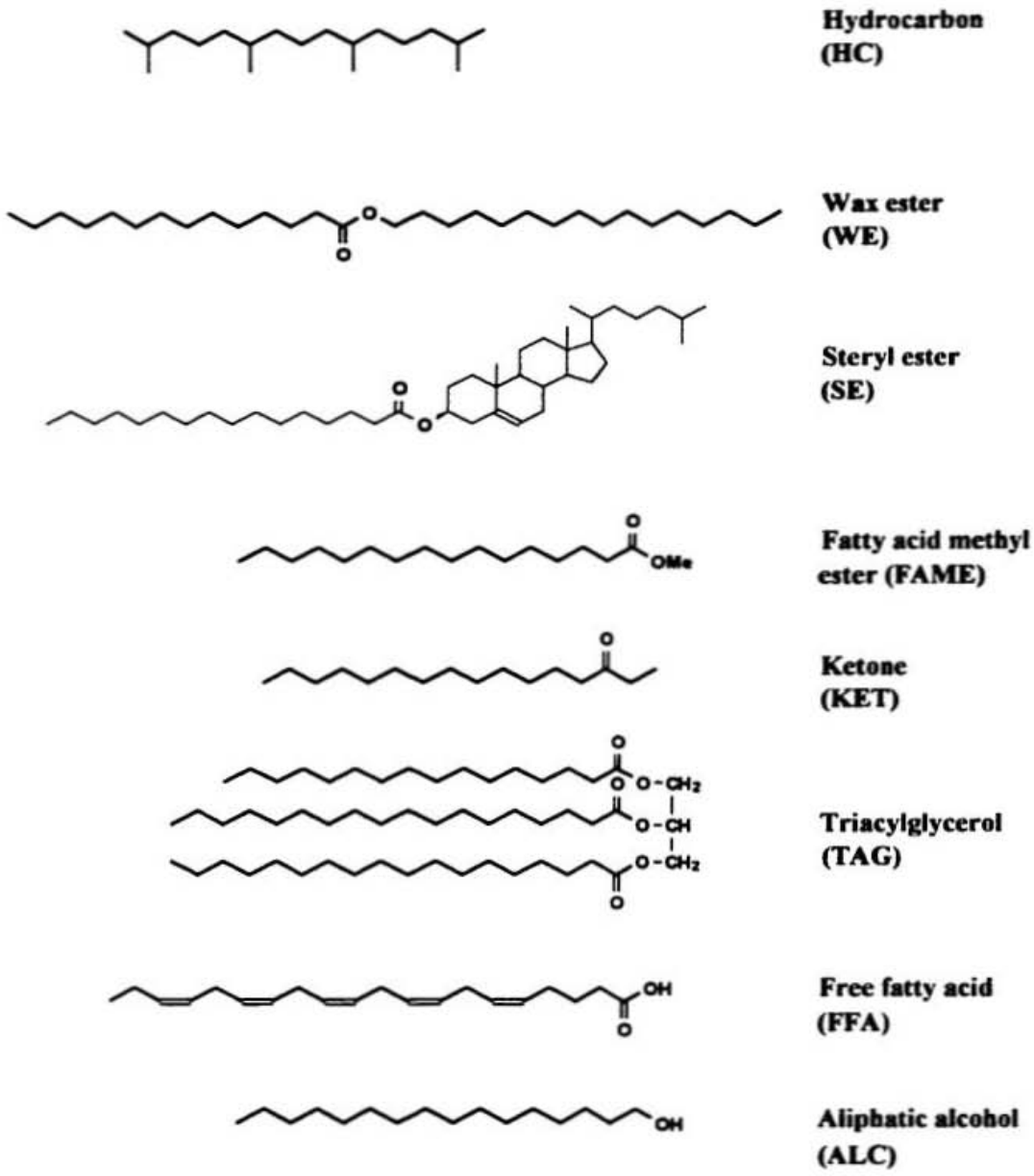


Figure 2.1 Representative compounds of lipid classes commonly found in marine samples.

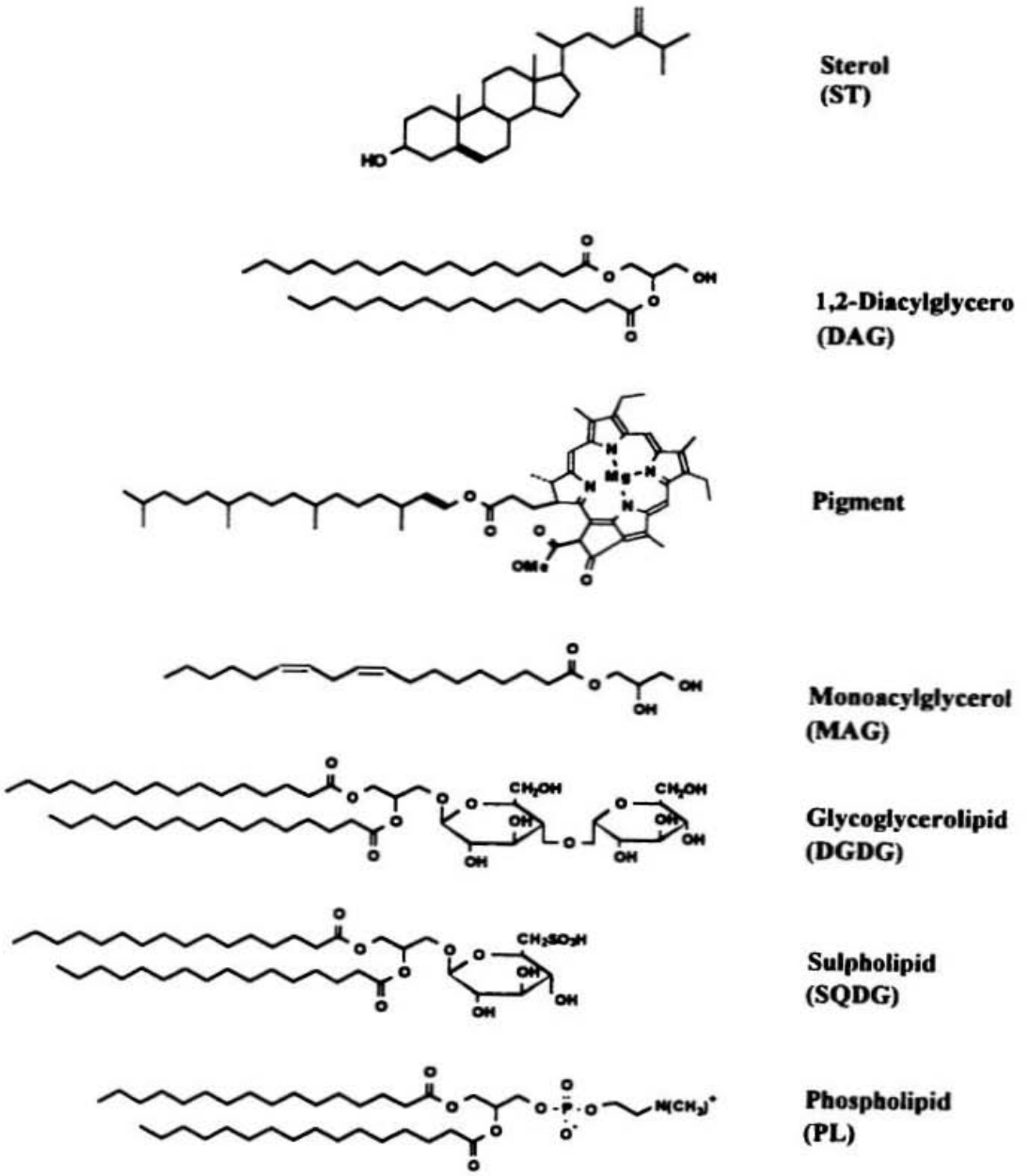


Figure 2.1, continued.

and including, the terminal methyl group to the nearest ethylenic bond. This system assumes that all ethylenic bonds are *cis* in configuration and methylene interrupted (Ackman, 1989). With non-methylene interrupted fatty acids, a Greek delta (Δ) is used to indicate the position of the double bond relative to the carboxyl group. For example, 20:2n-6 and 20:2Δ5,11 represent 11,14-eicosadienoic acid and 5,11-eicosadienoic acid, respectively. *Iso-* (*i-*) and *anteiso-* (*ai-*) indicate methyl branchings of the terminal end of the acid. Examples of structures and nomenclature are given in Figure 2.2. Fatty acids containing two or more double bonds are known as polyunsaturated fatty acids (PUFA). In higher animals, some of these PUFA, for example 20:5n-3 and 22:6n-3, are referred to as essential fatty acids because the animal has an absolute requirement for the fatty acid but is unable to synthesize the fatty acid itself.

2.2 Sources of Fatty Acids

2.2.1 Bacteria

The fatty acid compositions of both aerobic and anaerobic bacteria are quite different from those of typical plants and animals. Marine bacterial fatty acids include saturated, *cis*-monounsaturated and methyl-branched fatty acids (Perry *et al*, 1979; Parkes and Taylor, 1983; Gillan and Sandstrom, 1985; Rajendran *et al*, 1991). In addition, PUFA, including 20:5n-3 and 22:6n-3, have been found in deep-sea and Antarctic bacteria (DeLong and Yayanos, 1986; Nichols *et al*, 1997). Another unusual group of fatty acids associated with bacteria are *trans*-monounsaturated fatty acids (Gillan *et al*, 1981), particularly *trans*-16:1n-7

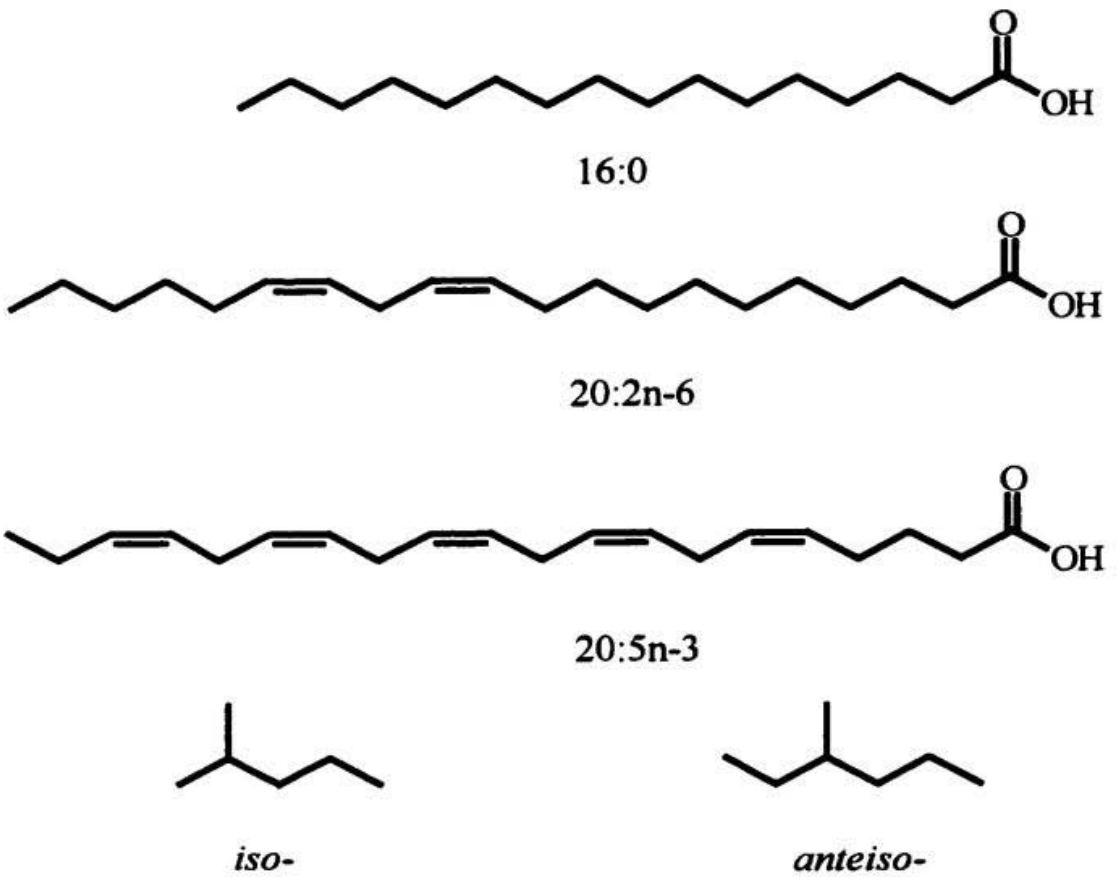


Figure 2.2 Nomenclature of fatty acids found in marine samples.

and *trans*-18:1n-7. These fatty acids have been found in prokaryotes but, like methyl-branched fatty acids, are predominantly found in bacteria (Keweloh and Heipieper, 1996).

Fatty acids of bacteria are usually confined to membrane lipids, as bacteria do not have storage fats such as TAG (Keweloh and Heipieper, 1996). Because of this structural role, the fatty acid composition of bacteria fluctuates much less than that of other organisms that contain storage lipids. This makes certain fatty acids, particularly methyl-branched and *trans* fatty acids, useful as biomarkers for bacteria (Bowman *et al*, 1991; Caudales and Wells, 1991; Guckert *et al*, 1991; Ringelberg *et al*, 1994; Zelles and Bai, 1994). Indeed, Keweloh and Heipieper (1996) state that " the identification of bacteria with the help of their fatty acid composition as a biochemical marker has become a routine method." It should be noted, however, that although individual fatty acids may be associated with bacteria, those fatty acids are also found in other organisms, and only a combination of them can be used as a general bacterial marker (Sargent *et al*, 1987).

2.2.2 *Phytoplankton*

Phytoplankton have a variety of fatty acid compositions, but, within each class, the compositions are similar. Viso and Marty (1993) and Kayama *et al* (1989) reviewed the fatty acid composition of several classes of microalgae. Both report a characteristic fatty acid composition of diatoms (Class *Bacillariophyceae*) of 16:1n-7, 16:0, 20:5n-3 and 14:0 with the unusual fatty acid 16:4n-1 commonly present, while dinoflagellates (Class *Dinophyceae*) usually contain the rare fatty acid 18:5n-3, as well as higher amounts of 16:0, 18:4n-3 and

22:6n-3. Green algae (Class *Chlorophyceae* and *Prasinophyceae*) have a fatty acid composition resembling that of terrestrial plants with much larger amounts of 18:2n-6 and 18:3n-3 than are found in other classes. It should be noted that these compositions are generalizations for photosynthetic organisms. Non-photosynthetic organisms do exist in some of these taxa and display very different fatty acid compositions. In addition, fatty acid compositions are highly dependent on culture conditions and stage of growth when harvested. Whatever the composition, this phytoplankton forms the base of the food web and, in the marine environment, is by far the major source of essential fatty acids, such as 20:5n-3 and 22:6n-3, for animals.

These characteristic fatty acid compositions often make it possible to designate certain fatty acids or ratios of fatty acids as biomarkers of various phytoplankton classes. For example, the fatty acid 16:4n-1 is very rarely encountered in any class of microalgae other than *Bacillariophyceae*. A value of the ratio of monounsaturated C_{16} fatty acids/saturated C_{16} fatty acids (16:1/16:0) above 1.6 (Bodennec *et al*, 1994) is also used to indicate the presence of diatoms. Claustre *et al* (1988-89) interpreted an increase in the value of the ratio of C_{16} fatty acids/C_{18} fatty acids (ΣC16/ΣC18) as indicative of increased diatom proportions. The fatty acid 16:4n-1 has also been proposed as a more specific marker of a toxic diatom, *Pseudo-nitzschia multiseries* (Parrish *et al*, 1991) due to the high levels (11% of total fatty acids) of 16:4n-1 that it contains, but Chapter 5 shows that there are even greater amounts of this acid in the closely related *Pseudo-nitzschia pungens*. Similarly, it was hoped that 18:5n-3 could be used as a biomarker for the toxic dinoflagellate *Alexandrium fundyense*, but

higher levels are found in another non-toxic dinoflagellate, *Scrippsiella trochoidea* (Chapter 6). The evidence suggests that fatty acid biomarkers should be applied only generally to the various microalgae classes.

2.2.3 Microzooplankton

Microzooplankton mainly consist of flagellated and ciliated protozoans, such as foraminiferans, radiolarians and tintinnids, in the size range of 20 - 200 μm (Libes, 1992). This group also includes copepod nauplii (the young stage of copepods) and cryptomonads. Claustre *et al* (1988-89) found both copepod nauplii and tintinnids (*Stenosemella ventricosa*) to contain high levels of PUFA, from 47 to 59% of the total. These PUFA were primarily composed of 20:5n-3 and 22:6n-3, as well as 18:5n-3 in the tintinnid. In particulate samples, biovolumes of microzooplankton, including ciliates, tintinnids and choanoflagellates, have also been found to correlate significantly ($P<0.05$) with amounts of 20:4n-3, 18:5n-3 and total pentaenes (Parrish *et al*, 1995). Skerratt *et al* (1995) stated that high levels of C_{18} PUFA (approximately 7%) and 22:6n-3 (7.5%) in water column particulates were characteristic of cryptomonads. However, one study of the fatty acid composition of two ciliates (Harvey *et al*, 1997) reports little or no PUFA in those organisms. These two ciliates, *Pleuronema* sp. and *Fabrea salina*, were fed a diet low in PUFA and their fatty acid composition reflected this.

2.2.4 Higher Animals

Higher animals, including bivalves and finfish, fulfil their fatty acid requirements both by uptake of material produced through primary production and by biosynthesis. However, most of these organisms, particularly marine fish, are unable to produce sufficient quantities of certain PUFA and rely totally on dietary sources of these essential fatty acids (Sargent, 1995). In marine fish, 20:5n-3 and 22:6n-3 are essential and frequently comprise greater than 20% of total fatty acids by weight (Joseph, 1982). Since these fatty acids are also essential to humans, marine fish are often viewed as an important source of these fatty acids in our diets.

Bivalves commonly contain unusual non-methylene interrupted dienoic (NMID) fatty acids. The function of these fatty acids is unknown, but their biosynthetic pathway has been elucidated (Zhukova, 1986; Zhukova, 1991). Radio-labelled acetate was administered to *Mytilus edulis* (the blue mussel), and after 12 or 24 hours fatty acid radioactivity was determined. All saturated and monounsaturated fatty acids were radio-labelled, but the only radioactive PUFA were 20:2 NMID and 22:2 NMID. The double bond locations of these fatty acids were determined to be 20:2Δ5,11, 20:2Δ5,13, 22:2Δ7,13 and 22:2Δ7,15. Zhukova reasoned that 20:2Δ5,11 and 20:2Δ5,13 were synthesized by insertion of a double bond at the Δ5 position in 20:1Δ11 and 20:1Δ13. Synthesis of the 22:2 NMID could then occur simply by chain elongation. These results suggest that *M. edulis* does possess a Δ5 desaturase enzyme but that it is very specific and only functions with monounsaturated fatty acids, as no radioactivity was found in the fatty acids 20:4n-6, 20:5n-3 and 22:6n-3. If NMID are the only

PUFA formed by *M. edulis*, then 20:4n-6, 20:5n-3 and 22:6n-3 are essential for that organism and probably for bivalves in general.

2.2.5 Sediments

Any organic material that is not recycled in the water column is ultimately deposited in the sediments on the sea floor. Marine sediments contain active populations of bacteria which cause further breakdown of organic material through respiration (Deming and Baross, 1993). As a result, sediments generally preserve only unreactive compounds. Saturated and monounsaturated fatty acids, particularly those with 16 and 18 carbon atoms, are most common but longer-chain saturates, attributed to higher plant sources, are also encountered (Haddad *et al*, 1992; Harvey, 1994; Colombo *et al*, 1997; Laureillard *et al*, 1997). PUFA usually comprise less than 5% of total fatty acids in sediments, probably due to both their reactivity and their incorporation in diets of benthic invertebrates and animals. Lipids of sediments are primarily composed of AMPL and PL with small amounts of FFA (Parrish, 1998). AMPL and PL in marine sediments have only been characterized in a few studies (Rajendran *et al*, 1991; Laureillard *et al*, 1997) and their fatty acid compositions are largely unknown.

2.3 Biosynthesis of Fatty Acids

All organisms are capable of synthesizing saturated fatty acids by basic condensation of two-carbon compounds (Zubay, 1993). The actual formation of saturated fatty acids is

quite complicated, involving a multienzyme complex, and is beyond the scope of this chapter. PUFA are formed by chain elongation and insertion of double bonds in these saturated fatty acids by the action of an appropriate enzyme. Phytoplankton are able to synthesize a variety of PUFA that higher organisms and terrestrial plants cannot because they possess different enzyme systems (Cook, 1985).

Generally, in both plants and animals, a double bond is first inserted into a saturated fatty acid at the Δ9 position, usually resulting in 16:1n-7 or 18:1n-9, depending on the saturated precursor. In animals, further desaturation must occur between the original double bond and the carboxyl end of the fatty acid with Δ6, Δ5 and, possibly, Δ4 desaturase enzymes. In terrestrial plants, desaturation occurs at the opposite end of the fatty acid, and double bonds are inserted at the Δ12 and Δ15 positions. Phytoplankton, however, are capable of desaturation on either side of the original double bond, allowing them to synthesize a greater variety of PUFA than animals or terrestrial plants (Cook, 1985). It should be noted that possession of the appropriate desaturase enzyme does not guarantee sufficient production of the corresponding fatty acid. An example is 22:6n-3 in humans, who possess the necessary desaturases and are capable of producing 22:6n-3 by chain elongation and desaturation, but cannot synthesize it in sufficient quantities to meet metabolic needs and therefore require a dietary source.

Fatty acids of the n-3 and n-6 families are essential, and animals are entirely dependent on plants, including phytoplankton, to provide them. Once an n-3 precursor is provided, the animal can produce a variety of fatty acids by chain elongation and desaturation. For example,

if 18:3n-3 is present in the diet, the animal can perform a Δ6 desaturation to yield 18:4n-3, followed by chain elongation to produce 20:4n-3 (Figure 2.3). If the animal possesses a Δ5 desaturase, it can then form 20:5n-3. In rat hepatocytes, to produce 22:6n-3, it has been demonstated that 20:5n-3 is elongated first to 22:5n-3 and then to 24:5n-3 (Voss *et al*, 1991). A Δ6 desaturase is then employed to form 24:6n-3 which undergoes chain shortening through β-oxidation to yield 22:6n-3. The inability of animals to alter the n-3 or n-6 position after it has been established is what led to the development of the n-3, n-6, n-9, etc. nomenclature. The animal can carry out chain elongation or desaturation but cannot change the "n" designation. Furthermore, some animals, such as marine fish, lack the Δ5 desaturase necessary to create 20:4n-6, 20:5n-3 and 22:6n-3, and therefore have a dietary requirement for those fatty acids, in addition to 18:3n-3 and 18:2n-6.

2.4 Methods of Analysis

2.4.1 Lipid Classes

2.4.1.1 Extraction

Before fatty acid analysis can be carried out, the lipids must first be extracted from the matrix in which they are encountered. Lipids are soluble in organic solvents, and their extraction from plant and animal tissues is commonly performed using a mixture of chloroform and methanol. Recently, Christie (1993) published a review of extraction procedures using other solvents, but in general either the "Folch" procedure (Folch *et al*,

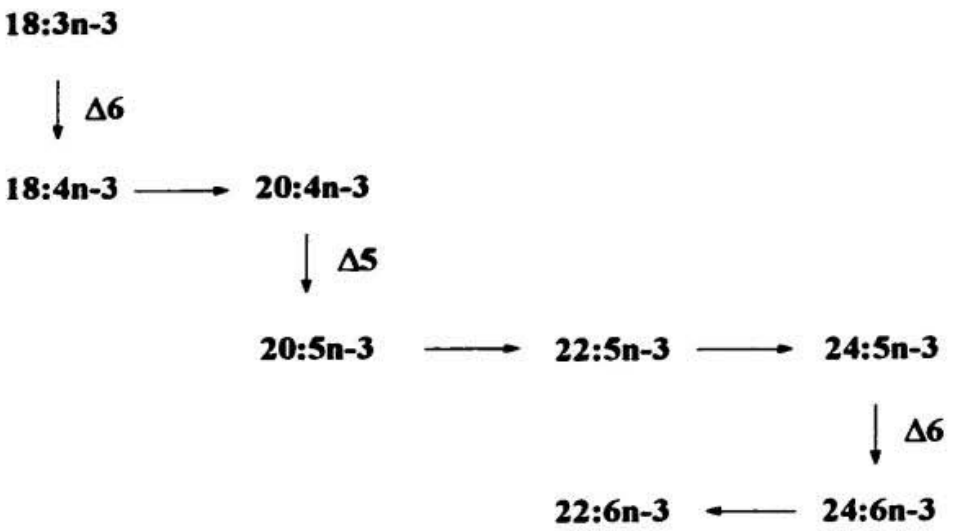


Figure 2.3 Schematic of the biosynthesis of 22:6n-3 from 18:3n-3 by chain elongation, chain shortening and desaturation.

1957) or the Bligh and Dyer (1959) method is used. Both employ mixtures of chloroform and methanol, the Folch procedure using a biphasic solvent system of chloroform:methanol:water 8:4:3 while Bligh and Dyer recommend a monophasic system employing a final chloroform: methanol:water ratio of 2:2:1.8. The Bligh and Dyer method was developed for use with wet tissues and Christie (1993) reports "good recoveries of the more important lipid classes" with this method, but the Folch procedure does seem to be more widely accepted.

Soxhlet extractions are commonly used in the extraction of lipids, particularly hydrocarbons, from wet sediment samples (Marvin *et al*, 1992; Bieger *et al*, 1997). Usually a non-polar solvent, such as dichloromethane, is refluxed through the sediment sample for several hours. No physical processes like sonication or homogenization with a blender are employed. A more polar solvent, such as methanol, can also be used in the extraction if polar lipids are to be recovered (Favaro, 1998).

Extraction of lipids according to any of the above methods may seem to be a straightforward matter, but several precautions must be taken to prevent lipid degradation and ensure accurate results (Christie, 1993). Firstly, samples should be extracted immediately after collection or frozen below -20 °C in solvent under a nitrogen atmosphere and in the dark (Sasaki and Capuzzo, 1984). During extraction, the samples should also be kept cold. These precautions are necessary to prevent activation of lipolytic enzymes and autoxidation. Some researchers have recommended deactivation of these enzymes by treating samples with boiling water prior to extraction (Berge *et al*, 1995). Diatoms contain particularly active lipolytic enzymes (Jüttner and Dürst, 1997) and may especially require this treatment. Many

laboratories also routinely add the antioxidant BHT (butylated hydroxy toluene) to samples. Both phytoplankton and bivalves contain the natural antioxidant tocopherol (Sigurgisladottir *et al*, 1993), so some protection against autoxidation is present naturally in such material.

2.4.1.2 Column Chromatography

Because neutral and polar lipids have different roles within the organism, there is much interest in separating lipid extracts into those two fractions (Delaunay *et al*, 1993; Ibeas *et al*, 1996; Soudant *et al*, 1996; Laureillard *et al*, 1997). Additional information can then be gained by examining the fatty acid composition of those fractions. A variety of methods exists to carry out this and more specific separations, but few separations are quantitative. Generally, a short column is prepared by packing a pasteur pipet with silica gel or Florisil and a relatively non-polar solvent, such as chloroform, is employed to elute neutral lipids, while a polar solvent, such as methanol, is used to elute the polar lipids (Christie, 1989). Deactivation of the silica gel or Florisil with water can also be employed to encourage elution of more polar lipids (Carroll, 1976). These column fractionations, however, are not as straightforward as commonly thought. For example, chloroform alone will not elute the more polar of the non-polar lipids, and Yang (1995) has shown that addition of small amounts of polar solvents such as methanol and formic acid to chloroform are necessary to elute free fatty acids from Florisil. In theory, it should also be possible to elute the poorly characterized AMPL fraction from samples simply using acetone as the solvent.

2.4.1.3 Thin-Layer Chromatography (TLC)

This technique is commonly used to separate lipid mixtures into individual classes (Henderson and Tocher, 1992). Samples are applied to silica gel-coated plates, and the plates are developed in appropriate solvents. The separated classes are then visualized and scraped from the plates for further analysis. Quantitation may be accomplished using scanning densitometry, gravimetry or absorbance measurements. Two dimensional TLC may also be employed to further separate lipids into subclasses (Henderson and Tocher, 1992).

2.4.1.4 TLC-Flame Ionization Detection (TLC-FID)

Routine separation, detection and quantitation of lipid classes is possible through the combination of TLC and FID. This system employs reusable silica gel-coated quartz rods on which the thin-layer chromatographic separation takes place, and quantitation is performed using the FID common in gas chromatography. After separation, the rods are simply scanned through the FID to detect the lipid classes. With the Iatroscan system, it is possible to carry out partial scans of the material on the rods, effectively increasing the rod length available for separation. Using partial scans and multiple solvent developments, it is possible to routinely separate 11 lipid classes including HC, WE/SE, methyl esters (ME), ketones, GE, TAG, FFA, ALC, ST, DAG, AMPL and PL. Methods for more detailed examinations of certain lipid classes have also been devised. For example, it is possible to further separate glycolipids in the AMPL fraction (Parrish *et al*, 1996a) and subclasses of PL may also be resolved (Tocher *et al*, 1985).

2.4.2 Fatty Acid Analysis

2.4.2.1 Fatty Acid Methyl Ester Formation

Fatty acids are routinely determined on capillary columns using gas chromatography (GC) by comparison of retention times with standards. However, to determine fatty acids in lipid classes, both qualitatively and quantitatively, it is first necessary to separate the fatty acid chains from the non-acyl backbone, often by saponification. Volatile derivatives of the free fatty acids must then be prepared to permit their chromatographic separation by GC. Fatty acid methyl esters (FAME) are commonly the derivative of choice and there are a variety of methods employed to prepare them. An initial saponification of the lipid extract is a possibility, but Christie (1982) claims that this is not necessary, and recommends a one step transesterification procedure. Transesterifications fall into two classes: those employing acidic catalysts and those employing basic catalysts. Acidic catalysts provide the advantage of esterifying free fatty acids in addition to performing the transesterification; they include methanolic hydrochloric acid, methanolic sulphuric acid and methanolic BF_3. Christie (1982) particularly favours a procedure involving methanolic hydrochloric acid and suggests that the use of BF_3 results in the production of artefacts and the loss of PUFA. Sodium methoxide in methanol is probably the most common of the basic catalysts, but like other basic catalysts it fails to esterify free fatty acids and should not be used when significant amounts of free fatty acids are expected. The American Oil Chemists Society (AOCS) has recommended Official Method Ce 1b-89 (1990), which is a combination of both acidic and basic techniques. Sodium methoxide is first employed to transesterify the lipid extract. Treatment with 12% BF_3 in

methanol then follows to esterify any FFA that are present.

2.4.2.2 Argentation-TLC

Silver ions reversibly form complexes with unsaturation centres in organic molecules (Dobson *et al*, 1995), and this property can be used to separate FAME into various groups according to double bond number and configuration. Usually, the technique employs a chromatography plate coated with silica gel that has been impregnated with $AgNO_3$. For the separation, a variety of solvent systems may be used. For example, Napolitano *et al* (1988) used benzene to separate the fatty acid methyl esters in an algal sample into six bands according to number of double bonds, while Rézanka (1996) used 9:1 hexane:diethyl ether as the developing solvent to produce the same separation. Lamberto and Ackman (1994) have even used this method to isolate a monoenoic trans band with benzene:hexane (2:1) as the solvent mixture. It is also possible to use a polar solvent mixture to first resolve fatty acid methyl esters with three to six double bonds, followed by a development of the monoenoic and dienoic components in a less-polar solvent (Nikolova-Damyanova, 1992). It should be noted that, with these methods, the stability of the complex formed by the acid and the silver nitrate increases with increasing number of double bonds, so there is no discrimination against highly unsaturated compounds (Nikolova-Damyanova, 1992). After the desired resolution is achieved, the esters are extracted with an appropriate solvent, often 1:1 hexane:chloroform (Napolitano *et al*,

1988; Lamberto and Ackman, 1994) or hexane:diethyl ether 1:1 (Rĕzanka, 1996).

In addition to solvent systems, there are several other factors concerning resolution that must be taken into account. The silver content of the silica gel is one such factor. Plates which contain $AgNO_3$ in a range of 5 to 20% are commonly used, but acceptable results have been obtained with as little as 0.5% and as much as 40% $AgNO_3$ (Nikolova-Damyanova, 1992). Temperature and humidity are also variables affecting any TLC separation. Finally, a visualizing agent, such as 2',7'-dichlorofluorescein or Rhodamine G6, must be sprayed on the plate after development for viewing under ultraviolet light (Nikolova-Damyanova, 1992).

2.4.2.3 GC-FID

Since GC is invariably used to separate fatty acid methyl esters, the FID with which practically all GCs come equipped has seen much use as well. Reduced carbon atoms are oxidized in the flame of the FID to generate electrons and ions. These charged species are captured by a collector resulting in an ion current that is then amplified and recorded. The FID offers the advantages of being rugged and inexpensive, as well as having a large linear range and a fast response (Skoog and Leary, 1992; Evershed, 1992a). The FID, of course, gives no information about the structure of the eluting compounds so FAME must be identified by comparison of retention times with known standards.

2.4.2.4 GC-Mass Spectrometry (GC-MS)

Mass spectral detectors are also commonly employed in GC analysis. This type of detection has the advantage of providing both quantitative and qualitative results, and its basic components consist of an ion source, a mass analyser and an ion detector. For lipid analyses, particularly fatty acids, typical modes of ionization include electron impact, chemical and electron capture (Murphy, 1993), while mass analyzers are commonly magnetic sector, quadrupole or ion trap (Evershed, 1992b). The ion detector employed is almost invariably some type of electron multiplier (Evershed, 1992b). Various combinations of ion sources and mass analyzers offer distinct advantages depending on the analyte.

Electron impact ionization involves the acceleration of electrons through a potential difference, commonly 70 eV. These electrons collide with analyte molecules and cause ionization. Extensive fragmentation usually results, which is useful in structure determination but molecular ions are not always produced. Chemical ionization is a similar but "softer" technique where the accelerated electrons in this case collide with an excess of reagent gas. The ionized reagent gas then ionizes the gaseous analyte through a charge exchange reaction, rather than a collision. Positive ions are commonly used but negative chemical ionization (or electron capture) can be employed with analytes containing electronegative atoms. Both types of chemical ionization produce little fragmentation and a prominent molecular ion.

Most magnetic sector instruments currently in use are double focusing analyzers, employing both electrostatic and magnetic analyzers. Ions usually pass through the electrostatic analyzer first where they are focused according to their kinetic energies. Ions

within the correct range of energies then pass to the magnetic analyzer where they are focused according to their *m/z* ratios. These double-focusing instruments provide high resolution and are capable of accurate mass measurements (Evershed, 1992b). Quadrupole analyzers are also very common and they consist of four parallel rods through which both a radiofrequency (RF) potential and a DC voltage are applied. This causes an oscillating electric field which in turn causes complex oscillations in ions entering the field. Ions of a very limited mass range are stable in this field and may pass through the rods. Separation of ions of differing *m/z* ratios is accomplished by varying the voltage applied to the rods. Quadrupole analyzers offer the advantages of being more compact, less expensive and more rugged than magnetic sector instruments (Skoog and Leary, 1992). Finally, ion trap analyzers consist of a ring electrode with an applied RF potential and two grounded end caps. Ions of an appropriate *m/z* value enter a stable circular orbit within the ring. Increasing the RF potential destablizes the lighter ions which are ejected through an end cap and detected. These ion trap analyzers are even more compact and inexpensive than quadrupole analyzers (Skoog and Leary, 1992). The quadrupole and ion trap analyzers with both electron impact and chemical ionizations are probably the most common in use today for fatty acid analysis.

2.4.2.5 Fatty Acid Derivatives for GC-MS

Fatty acid derivatives for GC-MS are of two basic types: those that involve derivatization of the double bonds ("on-site derivatization") and those that form derivatives of the carboxylic acid functionality ("remote group derivatization") (Schmitz and Klein,

1986). The most common technique of "on-site" derivatization involves oxidation of the bonds to vicinal diols (Evershed, 1992b). The diols may then be further derivatized to produce volatile compounds necessary for GC-MS. These derivatives include TMS ethers and dimethoxy and isopropylidene adducts (Ratnayake and Ackman, 1989). It is also possible to omit the diol derivatization and simply form epoxide or dimethyl disulfide adducts at the double bond (Ratnayake and Ackman, 1989). Hydrazine has also been used to partly reduce PUFA to monoenes so that simpler fragmentation patterns are produced (Christie, 1982). All of these derivatives, however, are only suitable for monoenoic and dienoic acids. Fragmentation patterns of more unsaturated fatty acids are far too complex for double bond location with "on-site" derivatization, and "remote group" derivatization must be employed.

The most popular "remote site" derivatives are those employing nitrogen compounds such as pyrrolidide or picolinyl esters (Evershed, 1992b). Both derivatives are used with unsaturated acids to stabilize the fragments and prevent double bond migration during ionization (Evershed, 1992b). Of these two, Christie (1998) suggests that picolinyl esters are the most diagnostic, and Harvey (1992) has published a useful review outlining the expected fragmentation patterns of various types of functionalities possible in fatty acids. The only drawback in the use of picolinyl esters is the lower volatility of these esters, preventing their elution within the maximum temperature range of a typical polyethylene glycol coated column. As a result, a more nonpolar column is required which

leads to poor chromatographic resolution. It should also be noted that published methods of picolinyl ester formation (Harvey, 1992; Christie, 1998) involve reaction of the free fatty acids, which necessitates an additional step of saponification of the lipid extract.

2.4.2.6 GC-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS)

Stable carbon isotope analysis of specific fatty acids is possible through the use of GC-C-IRMS and Scrimgeour (1997) has recently reviewed the application of that technique to fatty acids. First, the fatty acids are methylated to produce FAME which are then separated by GC. As each FAME elutes, it passes through a micro-furnace where it is oxidized to CO_2. The CO_2 then enters the MS where its *m/z* ratio is determined and a ratio of $^{13}C/^{12}C$ in the fatty acid is produced. This ratio is compared to that of a reference gas and reported as a δ value, defined in the following equation:

$$\delta = \frac{1000 \text{ X (isotope ratio of sample - isotope ratio of standard)}}{\text{(isotope ratio of standard)}}$$

Because the range of natural variation in isotope ratios is small, the difference is multiplied by 1000 to produce a value in parts per thousand or ‰.

Carbon isotope ratios of individual compounds are useful in that they can provide information about carbon sources (Fang *et al*, 1993; O'Malley *et al*, 1994) and biosynthetic pathways (Monson and Hayes, 1980). For example, terrestrial plants, employing isotopically light atmospheric CO_2 in photosynthesis, are generally depleted in ^{13}C and have δ values near

-30‰ (Libes, 1992). However, marine phytoplankton photosynthetically fix heavier dissolved CO_2, producing more enriched products with δ values closer to -20‰ (Libes, 1992). These source specific $^{13}C/^{12}C$ ratios may be used to determine the origin of specific fatty acids. Kinetic fractionation of isotopes, occurring when an isotopically lighter substrate reacts faster than a heavier substrate producing ^{13}C-depleted products, may also offer some insight into the biosynthetic pathways of particular compounds (Monson and Hayes, 1980).

2.5 Thesis Objectives

The purpose of this study was to assess the methodology currently in use for fatty acid and lipid class analyses, including sample preparation, handling and derivatization. Where the methodology was found less than optimum, new or more appropriate techniques have been proposed and tested. These techniques were used to determine the structure of several unusual fatty acids and were applied to several sample sets to demonstrate the usefulness of fatty acid analyses in environmental studies, with particular emphasis on their use as biomarkers. The specific objectives were to:

1) Evaluate Folch *et al* (1957), Bligh and Dyer (1959) and Soxhlet extraction methods based on lipid yields of real samples.

2) Determine the optimal method of FAME formation from acyl lipids.

3) Investigate the effects of lipolytic enzyme deactivation in diatoms.

4) Evaluate the usefulness of picolinyl ester derivatization-GC-MS in structure determinations and to develop a rapid method of their formation from FAME.

5) Develop a column chromatography method capable of separating lipids into neutral, AMPL and PL fractions and use this method to characterize the AMPL and PL fractions in sediments.

6) Develop a method of calculating absolute fatty acid concentrations from acyl lipid data and fatty acid proportions.

7) Apply fatty acids as biomarkers in two biogeochemical studies in very different cold water environments.

8) Evaluate the usefulness of fatty acids as biomarkers of specific phytoplankton species.

Chapter 3 - FATTY ACID DETERMINATION IN COLD WATER MARINE SAMPLES

3.1 Introduction

Fatty acid analysis is of utmost importance in a variety of fields, including biochemistry, oceanography, geochemistry and aquaculture. This widespread use has led to the development of diverse techniques for their analyses, including, for example, various extraction, fractionation and methylation procedures. Ideally, standard methods would be adopted by all groups conducting fatty acid research. However, it is difficult to convince others to practice these standard methods. For example, the American Oil Chemists' Society has published an Official Method (CE b-89, 1990) for the methylation of fatty acids, but few groups actually follow this procedure. The problem is that much faster and simpler methods exist that seemingly accomplish the same purpose. To allow comparisons to be made of results acquired by different techniques, some knowledge of the effects on fatty acid recovery is required.

Accurate determination of fatty acid composition involves several steps, including at least lipid extraction and fatty acid methylation, assuming instrumental parameters have been optimized. This chapter will describe the optimization of extraction and methylation procedures for highly unsaturated marine samples. Additional procedures, such as fractionation of extract, removal of contaminants and formation of derivatives for mass spectrometry, are often performed as well, and new methods for those steps will be described

and critically evaluated. Finally, a simple method to allow estimation of fatty acid concentrations from acyl lipid data will be discussed and applied to marine samples. In most cases, results of all comparisons will be evaluated by application to three typical marine samples: *Nannochloropsis* sp. (green algae), *Mytelis edulis* (bivalve) and sediments from the ocean floor.

3.2 Experimental

3.2.1 Materials and Marine Samples

All chemicals and solvents employed in lipid and FAME analysis were of analytical or chromatographic grade. All solvents were supplied by Fisher Scientific (Ottawa, ON), while all other reagents, including lipid and FAME standards, were obtained from Sigma-Aldrich (Oakville, ON)

Algal samples of *Nannochloropsis* sp. and *Isochrysis galbana* were taken from cultures in logarithmic phase grown at the Ocean Sciences Centre (OSC). Samples of *Mytelis edulis* were also taken from stocks maintained at the OSC. Sediment samples were collected with a box corer near Hickman's Harbour in Trinity Bay, NF.

3.2.2 Statistical Analysis

The SigmaStat software package (Version 2.03) was used to perform all statistical tests. Statistically significant differences were determined using the Student's t-test when comparing only two sample sets. This test compares the mean and standard deviation of the

two samples and generates a P value which represents the probability that the two sets are significantly different. For example, a P value less than 0.05 indicates that the probability that the differences between the two sets are due to chance is less than 5%. P values less than or equal to 0.05 were interpreted as representing statistically significant differences. Significant differences among three or more sample sets were determined using one way analysis of variance combined with the Tukey Test (an all pairwise multiple comparison). This test is more conservative than simply applying multiple t-tests and also generates P values for each combination of sample pairs.

3.2.3 *Extraction*

3.2.3.1 Modified Folch *et al* (1957) Extraction

Samples of organic and inorganic particulate matter, referred to as seston (Shumway *et al*, 1987), are typically extracted following a modified Folch extraction such as that described in Parrish (1999). Samples containing approximately 100 mg of lipid were collected on glass fibre filters and placed in test tubes containing 3 mL of 2:1 chloroform:methanol. They were then ground with a stainless steel rod and 1 mL of 2:1 chloroform:methanol and 0.5 mL of water were added. The mixture was sonicated for four minutes to further break up cell membranes and centrifuged at 4000 rpm for two minutes. The lower chloroform layer was then removed and stored below 0 °C under nitrogen. At least three washes with 3 mL of chloroform were made of the mixture with sonication and centrifugation repeated each time.

3.2.3.2 Bligh and Dyer (1959) Extraction

This method was based on the theory that optimum extraction should occur when tissues are homogenized with a monophasic mixture of chloroform, methanol and water. This procedure incorporates the water present naturally in the tissues and was developed for animals such as fish that contain approximately 80% water. To each 5 g sample, 5 mL of chloroform and 10 mL of methanol were added and the mixture was homogenized with a blender for two minutes. The homogenate was then filtered through a Buchner funnel and the filtrate was collected. The tissue and the filter were rehomogenized with another 5 mL of chloroform and the mixture was again filtered. Both portions of filtrate were transferred to a separatory funnel, 5 mL of water were added and the funnel was shaken to ensure complete mixing. The layers were allowed to separate overnight and the bottom chloroform layer was collected.

3.2.3.3 Soxhlet Extraction

Soxhlet apparatus is generally used with sediment samples and a variety of solvents can be employed. Favaro (1998) describes a method where approximately 10 g of dried sediment was placed in the thimble and refluxed overnight with 100 mL of 9:1 methylene chloride:methanol. The solvent containing the lipids was collected and reduced in volume using a rotary evaporator.

3.2.4 Quantitation of Lipid Classes

3.2.4.1 Total Lipid Class Separation

Lipid classes were determined using the Iatroscan FID system according to the method described in Parrish (1999). Briefly, samples of lipid extract were applied to the Chromarods which were then developed twice in 99:1:0.05 hexane:diethyl ether:formic acid for 25 and 20 minutes. The rods were then scanned to just after the ketone peak. The second development was for 40 minutes in 80:20:0.1 hexane:diethyl ether:formic acid. Rods were then scanned to just after the DAG peak. Finally, the rods were developed twice in acetone (2 x 15 minutes), followed by a double development in 5:4:1 chloroform:methanol:water (2 x 10 minutes). The entire length of the rods was then scanned. This yielded three partial chromatograms which were joined to produce one full chromatogram.

3.2.4.2 Glycolipid Separation

Parrish *et al* (1996a) developed a procedure to separate monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulphoquinovosyl diacylglycerol (SQDG) from each other and from neutral lipids, chlorophyll *a*, MAG and PL. Chromarods containing the samples were developed for 40 minutes in 3:2 chloroform:acetone and then scanned until just after the MGDG peak. Rods were then redeveloped in 98:2 acetone:formic acid for 30 minutes, followed by a full scan. The two separate scans were then combined as above to yield an entire chromatogram.

3.2.4.3 PL Separation

Tocher *et al* (1985) have developed a method to separate PL subclasses in Atlantic herring. With this method, it was possible to separate phosphatidylcholine (PC), phosphatidylethanolamine, lyso-phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol. Briefly, the method involves an initial double development in acetone for 12 and 10 minutes, followed by a partial scan until just behind the neutral and acetone-mobile polar lipid (AMPL) peak. The rods were developed twice in 60:30:3 chloroform:methanol:water for 35 and 30 minutes. The entire length of the rod was then scanned. Only this second scan yielded a useful chromatogram, as no separation took place in the first development. This first development simply served to move all lipids except PL from the origin so that they could be burned off the rods.

3.2.5 Fractionation of Lipid Extract

Lipids were fractionated using a column chromatography method based on the procedure of Yang (1995) developed for recovery of neutral lipids. A small amount of pre-combusted glass wool was placed in the tapered end of a pasteur pipet and the pipet was packed with approximately 0.8 g of silica gel. The silica gel had been activated by heating at 110 °C for 1 hour. The column was then rinsed with two bed volumes of methanol and chloroform. Approximately 5 mg of lipid extract was placed at the top of the column and neutral lipids were recovered with two bed volumes (approximately 6 mL) of 98:1:0.5 chloroform:methanol:formic acid at a flow rate of approximately 1 mL min^{-1}. AMPL were

eluted with 2 bed volumes of acetone. One bed volume of chloroform was then passed through the column to return it to a more neutral polarity and PL were eluted with 2 bed volumes of methanol. Up to 20 mg of lipids were fractionated in this way using proportionally greater solvent volumes. It should be noted that with highly pigmented samples, such as sediments, to elute the neutral lipid fraction, it was necessary to use only 1 bed volume of the first solvent system to prevent the collection of some pigmented AMPL species in the neutral lipid fraction.

3.2.6 FAME Formation

3.2.6.1 BF_3/Methanol Catalyst

A rapid method of transesterification of lipid extracts to yield FAME employs 10-14% BF_3 in methanol (Morrison and Smith, 1964). An aliquot of lipid extract was evaporated to near dryness and 0.5 mL of hexane and 1 mL of BF_3/methanol reagent were added. The mixture was shaken and then heated at 80-85 °C for 1 hour. The samples were allowed to cool, 0.5 mL of water was added and the samples were again shaken. Two mL of hexane were added and the mixture was shaken, then centrifuged. The upper hexane layer, containing the FAME, was removed and concentrated.

3.2.6.2 HCl Catalyst

Based on the method of Christie (1989), 2 mL of acetyl chloride were added very slowly to 18 mL of methanol to make methanolic HCl. A portion of the lipid extract was

evaporated to near dryness and 1 mL of hexane and 2 mL of HCl/methanol mixture were added. The samples were then heated for 2 hours at 80 °C. Samples were allowed to cool and 3 mL of 5% aqueous NaCl were added. Two mL of hexane were added, the sample was shaken and the hexane was withdrawn. This hexane wash was repeated and both washes were pooled. The hexane fraction was washed with 2 mL of $NaHCO_3$ and dried over anhydrous Na_2SO_4.

3.2.6.3 H_2SO_4 Catalyst

Based on the method of Keough and Kariel (1987), samples were evaporated to near dryness and 2 mL of 6% H_2SO_4 in methanol were added. Five mg of the antioxidant hydroquinone were also added and the mixture was then heated at 70 °C for 5 hours. The samples were allowed to cool and 1.0 mL of water and 1.5 mL of hexane were added. The mixture was shaken and centrifuged and the upper hexane layer was collected.

3.2.6.4 American Oil Chemists' Society (AOCS) Official Method

The lipid extract was evaporated to near dryness and 0.5 mL of 0.5 mol L^{-1} NaOH in methanol was added. The mixture was heated at 100 °C for 7 minutes and was then allowed to cool. One mL of 10% BF_3 in methanol was added and the mixture was heated at 100°C for 5 minutes. The sample was again allowed to cool, one half mL of iso-octane was added and the mixture was shaken for 30 seconds. Two mL of saturated NaCl solution was added and the solution was shaken and centrifuged. The upper iso-octane layer was then collected. The

lower methanol/water phase was then extracted again with a further 0.5 mL of iso-octane and the iso-octane fractions were pooled.

3.2.6.5 Sodium Methoxide Catalyst

Bannon *et al* (1982) examined the equilibria involved in the base-catalyzed methylation of lipids and reviewed the relevant literature to develop the following rapid procedure. The lipid extract was evaporated to near dryness and two mL of 0.25 mol L^{-1} sodium methoxide in 1:1 methanol:diethyl ether were added. The mixture was covered with nitrogen and heated at 100 °C for 30 seconds. The sample was cooled, 1 mL of iso-octane and 5 mL of saturated NaCl were added and the mixture was shaken vigorously for 15 seconds. The upper iso-octane layer was then collected.

3.2.7 Hydrocarbon Clean-up with Column Fractionation

Approximately 20% of samples collected on ships were contaminated with hydrocarbons, likely a result of fuels and lubricants present on board. These hydrocarbons elute on an Omegawax column with FAME and are capable of obscuring FAME peaks. Because of this, it was necessary to remove these hydrocarbon contaminants before GC analysis. As with the lipid fractionation, this was accomplished with column chromatography using a pasteur pipet packed with silica gel in a modification of the procedure recommended by Christie (1989). The silica gel had been activated by heating at 110 °C for 1 hour. The column was rinsed with one bed volume of chloroform and hexane. The FAME sample was

then placed at the head of the column and one bed volume (approximately 3 mL) of iso-octane was used to elute the contaminating hydrocarbons. The FAME were then recovered with two bed volumes of 80:20 hexane:diethyl ether.

3.2.8 Argentation-TLC

The high molecular mass of picolinyl derivatives necessitates the use of a non-polar GC column that can be operated at high temperatures and this results in poor separation. To improve GC separations, the FAME were first separated according to bond number using argentation-TLC before picolinyl ester derivatization. Silver nitrate-impregnated plates were prepared by dipping silica gel-coated plates (14 cm in length with a coating thickness of 250 μm) in 20% $AgNO_3$ in acetonitrile (w/v). Plates were activated for 1 hour at 110 °C immediately prior to their use. Approximately 10 μg of FAME were applied in a concentrated spot using a Hamilton syringe. Following the method of Rězanka (1996), plates were then developed in a closed chamber in 90:10 hexane:diethyl ether until the solvent front reached the top of the plate after approximately 20 minutes. The plates were allowed to dry and were sprayed with a solution of 0.1% 2',7'-dichlorofluorescein in ethanol. Spots were visualized under UV light and scraped from the plate. The esters were recovered from the silica by extraction with 1:1 hexane:diethyl ether.

3.2.9 Picolinyl Ester Formation

Several methods (Christie, 1989; Harvey, 1992; Christie, 1997) for formation of picolinyl esters have been reported but all involve the reaction of free fatty acids, making an initial saponification step necessary. According to Hamilton *et al* (1992), saponification is accomplished by reaction of the lipid extracts with 0.5 mol L^{-1} NaOH in methanol at 70 °C for half an hour. After saponification, Harvey (1992) recommended reaction of approximately 100 µg of free fatty acids with 1.0 mL of thionyl chloride to form acid chlorides. The mixture was left to stand at room temperature for 1 minute and the excess thionyl chloride was evaporated under a stream of nitrogen. One mL of a 1% solution of 3-(hydroxymethyl)pyridine in acetonitrile was then added, shaken and centrifuged. Harvey (1992) stated that the mixture could then be injected directly into the GC. Christie (1989) recommended a similar method where FFA are first reacted with trifluoroacetic anhydride at room temperature for 30 minutes to form anhydride derivatives, followed by reaction with 3-(hydroxymethyl)pyridine.

A new method of picolinyl ester formation by transesterification was developed. Approximately 10 mg of freshly cut Na was dissolved in 10 mL of 3-(hydroxymethyl)pyridine by sonication. One mL of this solution was added to the FAME sample that had been concentrated to near dryness. Twenty beads of molecular sieve (Type 3A) were added, the mixture was covered with N_2 and heated at 80 °C for 1 hour. The mixture was allowed to cool and 2 mL of hexane were added to extract the picolinyl esters.

3.2.10 Chromatographic Conditions

3.2.10.1 GC-FID Analysis of FAME

FAME were analyzed using a Varian 3400 GC equipped with a temperature-programmable injector and a Varian 8100 autosampler. A flexible fused silica column (30 m x 0.32 mm ID) coated with Omegawax (Supelco, Mississauga, ON), especially designed for separation of PUFA, was used for general FAME separation. Hydrogen (flow rate 2 ml min^{-1}) was used as the carrier gas, and the gas line was equipped with an oxygen scrubber. The following temperature ramp was employed: 65 °C for 0.5 minutes, hold at 195 °C for 15 minutes after ramping at 40 °C min^{-1} and hold at 220 °C for 0.75 minutes after ramping at 2 °C min^{-1}. Helium (make-up gas) and air (combustion) had flow rates of 30 ml min^{-1} and 300 ml min^{-1}, respectively. The FID was isothermal at 260 °C, while the injector was programmed to rise to 250 °C at a rate of 200 °C min^{-1} after holding at 150 °C for 0.5 minutes. The injector was then held at 250 °C for 10 minutes. Autoinjection, with a sample size of 1 µl and a solvent plug size of 0.8 µl, was used. FAME were identified by comparison of retention times with known standards (PUFA1, PUFA2 and 37 Component FAME Mix).

Peak integration and quantitation were performed with the Varian Star Chromatography Software (Version 4.02). A signal/noise ratio of 3 and a tangent height of 100% was used. The initial peak width was 4 seconds. Peak area reject values were adjusted in each chromatogram to yield a minimum peak area of 0.10% of total peak areas. Events to inhibit integration were also used in chromatograms to force baseline adjustments and to ignore any common phthalate peaks which had been located with GC-MS.

3.2.10.2 GC-MS Analysis of FAME

The identity of several PUFA was confirmed by GC-MS. Both a Hewlett-Packard 5890/5971A GC-MS and a Varian 3400/Saturn GC-MS were employed. The same column and temperature programme as described above was used. Helium was used as the carrier gas with a flow rate of 2 mL min^{-1}. Both interfaces to the mass analyzers were maintained at 280 °C. The injector of the Hewlett-Packard was isothermal at 250 °C, while the injector of the Varian model followed the same temperature programme as employed with the GC-FID. Both mass analyzers also used a 70 eV ionization potential and scanned over a mass range of 50-500 m/z.

3.2.10.3 GC-MS Analysis of Picolinyl Esters

Picolinyl esters were analyzed on the Hewlett-Packard 5890/5971A GC-MS only. A 70 eV ionization potential was used and the mass range was again 50-500 m/z. The esters were separated on a CP-Sil 5CB coated column (25 m x 0.25 mm ID) using the following temperature program: 190 °C for 0.5 minutes followed by a ramp to 295 °C at a rate of 3.0 °C min^{-1} and hold at 295 °C for 9.5 minutes. Helium was employed as the carrier gas at a flow rate of 2 mL min^{-1}. The injector was isothermal at 250 °C and the mass analyzer interface was held at 280 °C.

3.3 Results and Discussion

3.3.1 Extraction Comparison

3.3.1.1 Modified Folch Procedure *vs* Soxhlet

Standard procedure in this laboratory which deals primarily with seston samples involves a Folch *et al* (1957) extraction. Several collaborative projects, however, provided samples already extracted by various other means so it became necessary to evaluate the efficiency of these other methods. Three samples of approximately 1 g each of the same sediment core were extracted following the modified method of Folch *et al* (1957). From the same sediment core, three samples of approximately 10 g were extracted using a Soxhlet extraction. While there were no significant differences in total lipids between the Folch and Soxhlet methods, on average there was a larger amount of lipid material recovered with the Folch technique and this greater concentration of lipid seems to be due to the significantly larger ($P=0.019$) amount of PL found in the Folch extraction (Table 3.1). The larger amount of PL extracted with the Folch method was likely due to the polarity difference in the extraction solvents. Nelson (1991) discusses the necessity of including polar solvents, such as methanol and water, in the extraction mixture to ensure complete extraction of polar lipids, as well as to ensure that the solvent penetrates through any polar regions surrounding hydrophobic lipids that may be present in membranes. The Soxhlet technique only employed a 9:1 dichloromethane:methanol solvent mixture and did not include any water, while the Folch method used an 8:4:3 chloroform:methanol:water ratio. The higher proportion of polar solvents employed in the Folch method likely increased the yield of polar PL. As well, there

Table 3.1 Variation in lipid class composition as concentration (μg g dry weight^{-1}) and proportion of total lipids of sediment samples with differing extraction technique (mean ± standard deviation, n = 3) .

	Folch		Soxhlet	
	concentration	%	concentration	%
Hydrocarbons	120 ± 110	7.4 ± 5.3	32.0 ± 4.6	2.59 ± 0.46
Steryl/Wax Esters	17 ± 15	1.05 ± 0.71	6.6 ± 4.8	0.55 ± 0.44
Ethyl Ketones	22.3 ± 1.9	1.47 ± 0.22	9 ± 12	0.75 ± 0.96
Methyl Ketones	6.1 ± 9.8	0.34 ± 0.53	0 ± 0	0 ± 0
Triacylglycerols	34 ± 58	1.8 ± 3.2	40 ± 27	3.3 ± 2.3
Free Fatty Acids	84 ± 23	5.7 ± 2.2	66 ± 13	5.28 ± 0.90
Alcohols	40.6 ± 3.3	2.69 ± 0.58	20 ± 17	1.6 ± 1.3
Sterols	35.3 ± 1.2 *	2.35 ± 0.53 *	45.5 ± 2.7	3.66 ± 0.21
Diacylglycerols	30 ± 20	1.8 ± 1.1	56 ± 30	4.4 ± 2.2
Acetone-Mobile Polar Lipids	685 ± 87	44.6 ± 3.0 *	737 ± 41	59.14 ± 0.79
Phospholipids	471 ± 81 *	30.8 ± 5.1 *	233 ± 30	18.7 ± 2.0
Total	1500 ± 290	100.00	1246 ± 85	100.00

* Data are significantly different ($P<0.05$) between the extraction techniques.

were also slightly, but significantly (P=0.004), less ST in the Folch extraction. Perhaps the Soxhlet extraction solvent had a polarity better suited to extraction of ST than that employed in the Folch technique.

Often, lipid class results are also expressed as weight percent of total lipids and results of the sediment extraction are presented in this manner in Table 3.1. Significant differences were observed in percent AMPL (P=0.001), in addition to ST (P=0.017) and PL (P=0.019). However, percent data are proportions and a large significant difference in one lipid class, such as PL, may actually create a difference in another class, in this case AMPL. For this reason, significant differences in percent results should be interpreted with caution. This is a particular problem with FAME data which are commonly reported as percent data.

Often extractions of lipids are carried out to determine FAME composition, so it was also useful to compare FAME yields of the two extraction methods (Table 3.2). FAME concentrations were determined by adding 1 mL of 10.22 ug mL^{-1} 23:0 FAME as internal standard to each extract immediately after extraction. Peak areas were then compared to that of the known standard. The Folch method yielded significantly higher concentrations of branched (P=0.035), saturated (P=0.010), monounsaturated (P=0.007) and total FAME (P=0.009). This higher FAME concentration is to be expected from an extraction procedure that produced significantly larger PL yields. However, it is certainly worth noting that a comparison of FAME results in terms of percent composition did not yield any significant differences. This is particularly important because, if a laboratory only requires FAME data as a proportion of total FAME, the most convenient method can then be employed. The lack

Table 3.2 Variation in fatty acid composition as concentration (μg g dry weight^{-1}) and proportion of total fatty acids of sediment samples with differing extraction techniques (mean ± standard deviation, n = 3).

	Folch		Soxhlet	
	concentration	%	concentration	%
Branched				
i-15:0	1.73 ± 0.19	2.11 ± 0.18	1.12 ± 0.24	2.19 ± 0.06
ai-15:0	4.70 ± 0.47	5.73 ± 0.40	3.11 ± 0.61	6.09 ± 0.10
i-16:0	1.03 ± 0.46	1.26 ± 0.54	0.46 ± 0.12	0.90 ± 0.06
ai-16:0	0.23 ± 0.02	0.28 ± 0.03	0.50 ± 0.19	0.95 ± 0.20
i-17:0	0.82 ± 0.08	1.01 ± 0.10	0.49 ± 0.14	0.95 ± 0.11
ai-17:0	1.00 ± 0.02	1.22 ± 0.09	0.58 ± 0.15	1.13 ± 0.17
Subtotal	9.5 ± 1.2 *	11.6 ± 1.2	6.3 ± 1.4	12.21 ± 0.28
Saturated				
14:0	8.4 ± 1.7	10.2 ± 1.6	6.2 ± 1.4	12.10 ± 0.57
15:0	3.12 ± 0.08	3.82 ± 0.31	2.05 ± 0.37	4.02 ± 0.11
16:0	19.5 ± 2.0	23.9 ± 2.3	10.0 ± 2.1	19.46 ± 0.46
17:0	0.35 ± 0.06	0.43 ± 0.06	0.46 ± 0.10	0.89 ± 0.03
18:0	5.00 ± 0.51	6.12 ± 0.73	2.02 ± 0.41	3.95 ± 0.06
20:0	1.54 ± 0.27	1.87 ± 0.25	1.05 ± 0.12	2.08 ± 0.20
22:0	2.69 ± 0.44	3.28 ± 0.42	2.18 ± 0.40	4.29 ± 0.31
24:0	4.93 ± 0.87	5.99 ± 0.76	4.00 ± 0.69	7.87 ± 0.35
Subtotal	45.6 ± 3.9 *	55.6 ± 2.2	27.9 ± 5.5	54.65 ± 0.29
Monounsaturated				
15:1	0.90 ± 0.05	1.10 ± 0.08	0.59 ± 0.08	1.17 ± 0.12
16:1n-9	0.00 ± 0.00	0.00 ± 0.00	0.91 ± 0.21	1.80 ± 0.36
16:1n-7	13.7 ± 1.4	16.8 ± 1.3	8.9 ± 1.9	17.32 ± 0.43
16:1n-5	1.41 ± 0.17	1.71 ± 0.17	0.83 ± 0.18	1.63 ± 0.06
18:1n-9	3.8 ± 1.4	4.7 ± 2.0	1.69 ± 0.35	3.32 ± 0.04
18:1n-7	4.34 ± 0.30	5.33 ± 0.64	2.32 ± 0.48	4.54 ± 0.03
18:1n-5	0.32 ± 0.06	0.39 ± 0.09	0.16 ± 0.02	0.32 ± 0.02
20:1n-11	0.43 ± 0.50	0.52 ± 0.60	0.40 ± 0.20	0.75 ± 0.29
20:1n-9	0.61 ± 0.63	0.76 ± 0.83	0.06 ± 0.10	0.14 ± 0.25
20:1n-7	0.48 ± 0.04	0.58 ± 0.04	0.25 ± 0.04	0.52 ± 0.21
Subtotal	26.1 ± 1.1 *	31.9 ± 2.8	16.1 ± 3.2	31.52 ± 0.07
Polyunsaturated				
16:2n-6	0.03 ± 0.05	0.04 ± 0.06	0.20 ± 0.05	0.39 ± 0.05
16:3n-4	0.00 ± 0.00	0.00 ± 0.00	0.39 ± 0.08	0.76 ± 0.01
18:2n-6	0.54 ± 0.81	0.63 ± 0.95	0.14 ± 0.04	0.28 ± 0.05
18:3n-3	0.21 ± 0.19	0.25 ± 0.22	0.10 ± 0.02	0.20 ± 0.00
Subtotal	0.77 ± 0.95	0.91 ± 1.11	0.83 ± 0.19	1.63 ± 0.10
Total	81.9 ± 4.3 *	100	51 ± 10	100

* Data are significantly different ($P<0.05$) between the extraction techniques.

of a significant difference among FAME results in terms of percent of total suggests that all acyl lipids had a very similar fatty acid composition.

3.3.1.2 Modified Folch Procedure *vs* Bligh and Dyer

Eight live samples of *Mytelis edulis* were individually removed from their shells, weighed and placed in pre-cleaned test tubes. Four of the eight were extracted individually following the modified Folch *et al* (1957) procedure, while the remaining four were extracted according to the previously outlined method of Bligh and Dyer (1959).

Significant differences were apparent in the concentrations of total lipid ($P=0.004$), methyl esters (ME) ($P=0.029$), ST ($P<0.001$) and PL ($P<0.001$) (Table 3.3). In all four cases, higher concentrations of total lipid were found in the Bligh and Dyer extracted material, with roughly 2.5 times more lipid recovered with that method. Such a huge difference in recoveries was entirely unexpected. Shaikh (1986) compared the recoveries of PL from porcine myocardial tissue using the original Folch *et al* (1957) procedure which employed blender homogenization and the Bligh and Dyer technique and found little difference (<10%) in yields. Indeed, one would expect the very similar solvent systems employed in the two methods to produce similar results. The large difference encountered in this study probably has much to do with the method used to break apart tissues and membranes, as evidenced by the much better recoveries of ST and PL. Homogenization using a blender as was employed with the Bligh and Dyer method seemed to be far more efficient at exposing lipids to the solvent than the sonication and grinding with a metal rod used in the modified Folch method. It should also

Table 3.3 Variation in lipid class composition as concentration (μg g wet weight^{-1}) and proportion of total lipids of *Mytelis edulis* samples with differing extraction techniques (mean ± standard deviation, n = 4).

	Bligh and Dyer		Folch	
	concentration	%	concentration	%
Hydrocarbons	27.7 ± 5.8	0.14 ± 0.05 *	74 ± 45	0.96 ± 0.54
Steryl/Wax Esters	4.0 ± 4.6	0.02 ± 0.03	1.4 ± 2.7	0.03 ± 0.07
Methyl Esters	400 ± 340 *	1.7 ± 1.3	31 ± 19	0.34 ± 0.16
Ethyl Ketones	340 ± 400	1.5 ± 1.6	36.5 ± 6.5	0.52 ± 0.29
Triacylglycerols	7000 ± 2300	32.0 ± 5.9	3500 ± 2700	36 ± 14
Free Fatty Acids	110 ± 81	0.52 ± 0.35	130 ± 130	1.23 ± 0.97
Alcohols	27 ± 54	0.11 ± 0.21	0 ± 0	0.00 ± 0.00
Sterols	1900 ± 210 *	8.8 ± 1.2	580 ± 210	7.1 ± 1.7
Diacylglycerols	250 ± 210	1.12 ± 0.82 *	355 ± 84	4.8 ± 2.4
Acetone-Mobile Polar Lipids	1200 ± 1000	5.5 ± 4.8	270 ± 120	3.07 ± 0.36
Phospholipids	10400 ± 1400 *	48.6 ± 5.1	3800 ± 1400	46 ± 12
Total	21600 4000 *	100.00	8800 4200	100.00

* Data are significantly different ($P<0.05$) between the two extraction techniques.

be noted that even after the tenth chloroform wash, several of the bivalve samples extracted with the modified Folch method still displayed a coloured solvent layer, indicating that more lipid material was being released with each sonication.

Examination of lipid class results as percent of total lipids also revealed some significant differences (Table 3.3). Higher proportions of FFA were found in the Folch extract, in addition to larger percents of HC (P=0.022) and DAG (P=0.025). Both FFA and DAG are indicative of lipolysis and suggest that the excessive sample handling involved in the repeated extraction and washing of the bivalve samples with the modified Folch method may have caused some lipid breakdown to occur. It was not possible to keep the samples ice-cold at all times with this method and perhaps the repeated exposure to room temperature contributed to lipolysis. This is an important effect to consider when large tissue samples are to be extracted with the Folch method of grinding and sonication. To summarize, the Bligh and Dyer technique, which was designed specifically for extraction of animal tissues, gave better recoveries of lipid from *M. edulis* than the modified Folch method that was developed for extraction of seston samples. If the modified Folch method must be applied to animal tissues, an initial blender homogenization, as used in the Bligh and Dyer technique, should be employed.

Absolute amounts of FAME recovered by the two techniques were not determined because, with such a huge difference in total lipids, FAME concentrations would also vary significantly between the two methods. However, FAME as percent of total were determined (Table 3.4). No significant differences were found among branched, saturated,

Table 3.4 Variation in fatty acid composition as proportion of total fatty acids and DMA of *Mytelis edulis* samples with differing extraction techniques (mean ± standard deviation, n=4).

Fatty Acid	Bligh and Dyer	Folch
Branched		
i-16:0	0.23 ± 0.04	0.21 ± 0.05
i-17:0	0.79 ± 0.05	0.71 ± 0.04
ai-17:0	1.37 ± 0.04	1.28 ± 0.17
i-18:0	0.20 ± 0.03	0.16 ± 0.03
Subtotal	2.59 ± 0.09	2.36 ± 0.19
Saturated		
14:0	3.11 ± 0.57	5.0 ± 2.0
15:0	0.65 ± 0.13	0.69 ± 0.01
16:0	11.32 ± 0.73	11.69 ± 0.67
17:0	0.76 ± 0.08	0.56 ± 0.07
18:0	2.92 ± 0.33	2.06 ± 0.46
Subtotal	16.2 ± 1.1	20.0 ± 1.9
Monounsaturated		
16:1n-9	0.18 ± 0.36	0.14 ± 0.29
16:1n-7	6.7 ± 1.1	10.1 ± 4.3
16:1n-5	0 ± 0	0.08 ± 0.16
17:1	0.38 ± 0.14	0.34 ± 0.09
18:1n-11	0.22 ± 0.02	0.21 ± 0.07
18:1n-9	0.74 ± 0.05	0.90 ± 0.28
18:1n-7	2.58 ± 0.30	2.60 ± 0.37
18:1n-5	0.17 ± 0.03	0.17 ± 0.03
20:1n-11	1.26 ± 0.48	1.14 ± 0.50
20:1n-9	2.28 ± 0.75	1.06 ± 0.44
20:1n-7	1.02 ± 0.39	0.38 ± 0.06
Subtotal	15.54 ± 0.45	17.1 ± 4.3
Polyunsaturates		
16:2	0.50 ± 0.04	0.72 ± 0.31
16:3n-4	0.09 ± 0.19	0 ± 0
16:4n-1	0 ± 0	0.43 ± 0.50
Polyunsaturates, continued		
18:2n-6	0.72 ± 0.08	0.81 ± 0.19
18:2n-4	0.15 ± 0.03	0.26 ± 0.05
18:3n-6	0.07 ± 0.05	0.06 ± 0.06
18:3n-4	0.07 ± 0.05	0.12 ± 0.09
18:3n-3	0.56 ± 0.07	0.75 ± 0.26
18:4n-3	0.80 ± 0.12	1.40 ± 0.57
18:4n-1	0 ± 0	0.04 ± 0.08
20:2Δ5,11	2.35 ± 0.33	1.89 ± 0.54
20:2Δ5,13	0.94 ± 0.38	0.76 ± 0.30
20:2n-6	0.50 ± 0.15	0.52 ± 0.12
20:3 NMI	0.35 ± 0.10	0.36 ± 0.02
20:4n-6	3.94 ± 0.31	3.14 ± 0.89
20:4n-3	0.05 ± 0.05	0.12 ± 0.08
20:5n-3	15.85 ± 0.80	17.1 ± 1.2
22:2Δ7,13	0 ± 0	0.04 ± 0.08
22:2Δ7,15	3.81 ± 0.35	3.42 ± 0.96
22:3	0.95 ± 0.08	1.07 ± 0.15
22:4n-6	0.26 ± 0.05	0.25 ± 0.08
22:5n-6	0.39 ± 0.06	0.32 ± 0.07
22:5n-3	0.94 ± 0.04	0.89 ± 0.23
22:6n-3	16.79 ± 0.36	15.0 ± 1.7
Subtotal	50.04 ± 0.70	49.5 ± 1.8
Dimethylacetals		
16:0 DMA	0.75 ± 0.04	0.50 ± 0.10
i-18:0 DMA	1.00 ± 0.04	1.03 ± 0.10
18:0 DMA	8.75 ± 0.39	7.3 ± 3.3
18:1 DMA	0.24 ± 0.02	0.15 ± 0.11
20:0 DMA	1.94 ± 0.14	1.60 ± 0.78
Subtotal	12.67 ± 0.51	10.6 ± 4.3
Total	99.61 ± 0.08	99.57 ± 0.10

monounsaturated or polyunsaturated FAME proportions isolated by the two extraction techniques. This implies that, while the Folch method may have been inferior in extracting all lipids, it does seem to have been effective in recovering a proportion of acyl lipids with structures representative of the total. It should also be noted that the *M. edulis* tissues were found to contain five different dimethylacetals (DMA). DMA are produced by methylation of plasmalogens, a unique type of lipid found in nervous tissue which contains a vinyl ether-linked acyl chain. Joseph (1982) reports the occurrence of plasmalogens in bivalves but this is the first report of *i*-18:0 DMA and 18:1 DMA in a bivalve.

3.3.2 Fractionation of Lipid Extracts

A variety of column chromatography methods for the separation of neutral and polar lipids exists (Carrol, 1976 and references therein) but most are just variations of a method where neutral lipids are eluted from silica gel using chloroform, followed by the elution of PL with methanol. However, critical analyses of these separations using TLC-FID are rare. Here, a method for separating lipid extracts into neutral lipids, AMPL and PL is evaluated in that manner.

Typical marine samples contain a variety of lipid classes, so to assess the success of the separation on real samples, the column chromatography technique was applied to samples of algae (*Nannochloropsis* sp.), animal tissue (*M. edulis*) and sediment samples. Figures 3.1, 3.2 and 3.3 display TLC-FID chromatograms of the fractionated extracts. With all three samples, both neutral and PL fractions predominantly contained the desired peaks, although

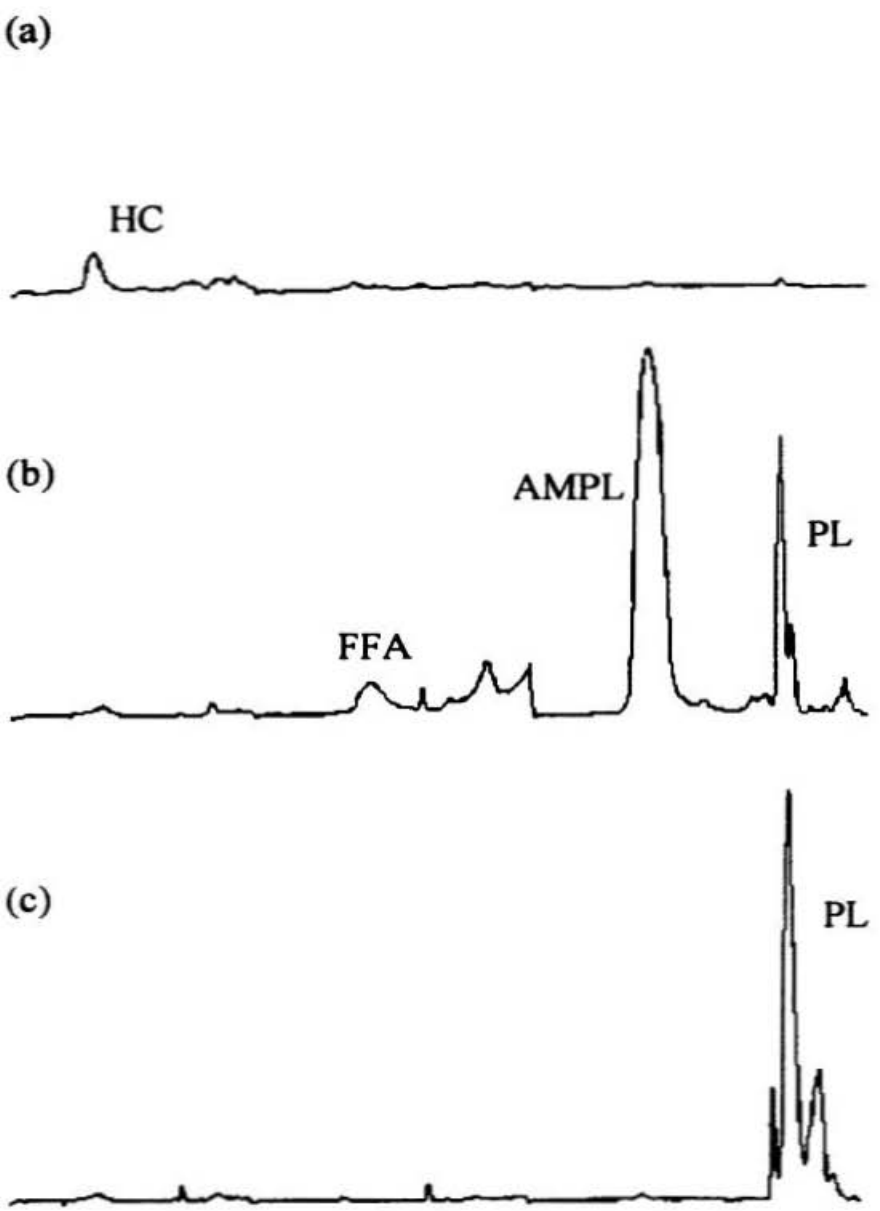


Figure 3.1 TLC-FID chromatogram of fractionated lipid extracts of *Nannochloropsis* sp. (a) Neutral fraction; (b) AMPL fraction and (c) PL fraction. Peak attentuation is the same for all chromatograms.

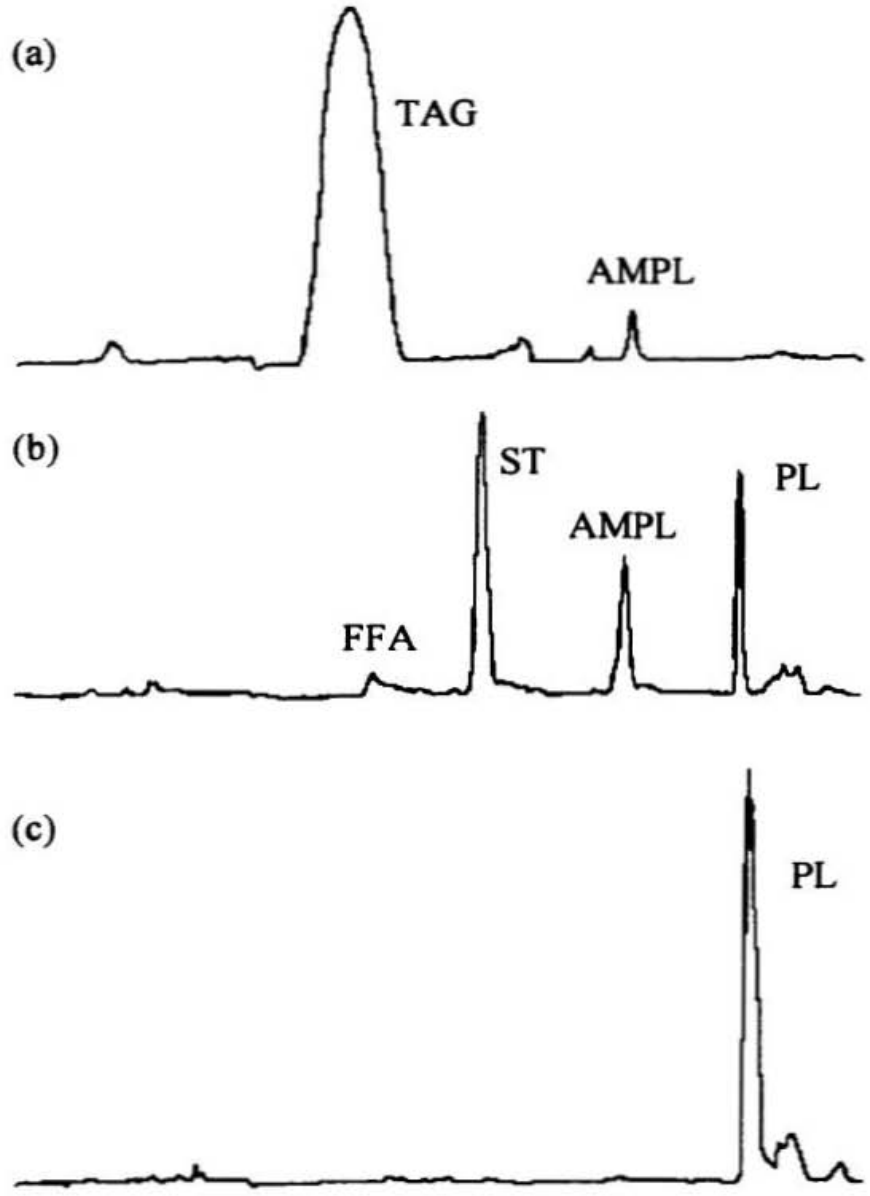


Figure 3.2 TLC-FID chromatogram of fractionated lipid extracts of *M. edulis*. (a) Neutral fraction; (b) AMPL fraction and (c) PL fraction. Peak attentuation is the same for all chromatograms.

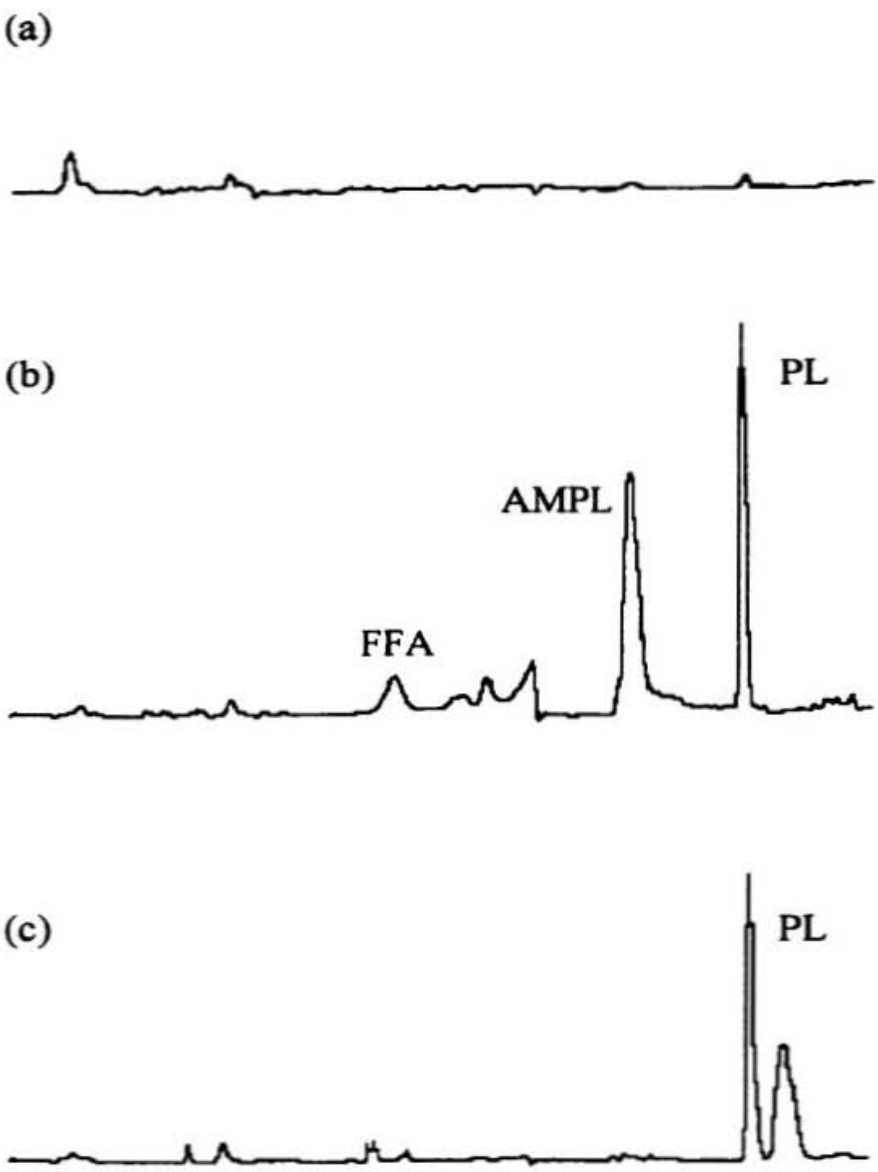


Figure 3.3 TLC-FID chromatogram of fractionated lipid extracts of sediments. (a) Neutral fraction; (b) AMPL fraction and (c) PL fraction. Peak attentuation is the same for all chromatograms.

a small amount of pigment (AMPL) was present in the neutral lipid fraction of *M. edulis*. In addition, the AMPL fraction in all three samples appeared to contain a portion of both the neutral and PL fractions. The peak labelled ST represents sterols and indicates that the lipid class eluted with acetone and in this separation is, by definition, an acetone-mobile polar lipid. The 98:1:0.5 chloroform:methanol:formic acid mixture was not polar enough to recover this rather polar lipid within the neutral lipid fraction. DAG are slightly more polar than ST and can also be expected to elute with acetone. However, DAG are rare in most marine samples and would normally make only a small contribution to total acyl lipids.

The other commonly encountered neutral lipid peak in the AMPL fraction was FFA. This was quite surprising as the 0.5% formic acid was included in the neutral lipid solvent specifically for the purpose of recovering FFA. This information, combined with the fact that PL are insoluble in acetone, suggests that both the FFA and PL peaks were the result of degradation of an AMPL compound on the silica gel column. Degradation of a glycolipid, for example, could be expected to produce a molecule of FFA and some more polar compound containing both a sugar moiety and an acylated fatty acid. However, for the purposes of determining fatty acid composition of each fraction, this degradation will have little impact on the utility of the column separation if changes in fatty acid structure with this breakdown are not apparent. In a study with sediments, where AMPL comprised more than 50% of total lipids, fatty acid methyl ester concentrations before and after fractionation were determined to be 74.6 ± 4.6 and 74.9 ± 6.1 μg g dry weight^{-1}, respectively. The only significant differences ($P<0.05$) in concentration were found among 16:1n-5, 17:0 and 20:1n-7 which

were only present in amounts less than 3% of total fatty acids. Concentrations of the major fatty acids remained unchanged. This suggests that alteration of fatty acid structure with breakdown of AMPL is not a concern.

Of the three fractions, recoveries of individual components in the neutral and AMPL fractions were close to 100 %. However, recoveries of PL were reduced on silica gel. PC was used as a model compound to determine the extent of this problem and only 73 ± 4% of the PC was recovered. In an attempt to improve recovery, the silica gel was deactivated with 20% water by weight for three hours. In one instance, this gave a recovery of 89% but results were variable and, on average, only 66 ± 22% was recovered. This was quite alarming as PC is often the major component in PL. In addition, many research groups assume recovery from silica gel is quantitative and may report fatty acid compositions of PL that are not accurate.

3.3.3 Methylation Techniques

To compare the efficiency of several different methods of FAME formation from lipid extracts, five 90 mL portions of *Isochrysis galbana* were filtered and extracted following the Folch procedure (Section 3.2.3.1). Each extract was made up to 7 mL and five 1 mL portions were transferred to vials containing 1 mL of 10.22 μg mL^{-1} 23:0 FAME as internal standard. Each group of five was derivatized according to the methods outlined in Section 3.2.6.

One way analysis of variance of the FAME concentrations (Table 3.5) revealed only a few significant differences in the results. Significant differences were apparent in monounsaturated (P=0.017), polyunsaturated (P=0.037) and total FAME (P=0.029) among

Table 3.5 Variation in fatty acid concentration (µg mL culture^{-1}) in cultures of *Isochrysis galbana* with differing derivatization procedures (mean ± standard deviation, n=4-5).

	Boron Trifluoride	Hydrochloric Acid	Sulphuric Acid	AOCS	Sodium Methoxide
Branched					
i-15:0	0.06 ± 0.04	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0.02
ai-15:0	0.01 ± 0.03	0.04 ± 0.07	0 ± 0	0.11 ± 0.02	0 ± 0
ai-16:0	0.09 ± 0.05	0.09 ± 0.03	0 ± 0	0.20 ± 0.00	0 ± 0
Subtotal	0.16 ± 0.06 b	0.13 ± 0.09 b	0 ± 0 c	0.32 ± 0.02 a	0.01 ± 0.02 c
Saturates					
14:0	3.10 ± 0.67	2.98 ± 0.49	2.95 ± 0.52	1.99 ± 0.30	2.07 ± 0.37
15:0	0.06 ± 0.02	0.03 ± 0.05	0.06 ± 0.01	0.04 ± 0.01	0.10 ± 0.03
16:0	1.49 ± 0.34	1.32 ± 0.29	1.38 ± 0.33	1.11 ± 0.21	1.03 ± 0.23
18:0	0.03 ± 0.03	0.05 ± 0.03	0.03 ± 0.02	0.03 ± 0.00	0 ± 0
Subtotal	4.7 ± 1.0	4.39 ± 0.80	4.42 ± 0.86	3.18 ± 0.52	3.21 ± 0.61
Monounsaturates					
16:1n-9	0.15 ± 0.09	0 ± 0	0 ± 0	0.55 ± 0.16	0 ± 0
16:1n-7	1.11 ± 0.24	1.03 ± 0.23	1.00 ± 0.19	0.84 ± 0.15	0.75 ± 0.12
18:1n-9	1.58 ± 0.25	1.54 ± 0.23	1.55 ± 0.26	1.48 ± 0.23	1.11 ± 0.23
18:1n-7	0.31 ± 0.03	0.32 ± 0.04	0.28 ± 0.03	0.16 ± 0.04	0.23 ± 0.11
Subtotal	3.16 ± 0.60 a	2.89 ± 0.49	2.83 ± 0.47	3.03 ± 0.47	2.10 0.30 b
Polyunsaturates					
16:2n-4	0.23 ± 0.03	0.26 ± 0.03	0.21 ± 0.03	0.15 ± 0.04	0.15 ± 0.04
16:3n-4	0.06 ± 0.02	0.09 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.11 ± 0.07
16:4n-1	0.07 ± 0.01	0.04 ± 0.03	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.03
18:2n-6	3.63 ± 0.59	3.39 ± 0.51	3.39 ± 0.55	2.97 ± 0.41	2.72 ± 0.36
18:3n-6	0.34 ± 0.06	0.32 ± 0.06	0.31 ± 0.06	0.27 ± 0.03	0.27 ± 0.04
18:3n-3	1.63 ± 0.19	1.51 ± 0.19	1.53 ± 0.20	1.36 ± 0.15	1.20 ± 0.11
18:4n-3	1.80 ± 0.25	1.62 ± 0.23	1.66 ± 0.25	1.43 ± 0.18	1.40 ± 0.17
20:2n-6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.06 ± 0.04
20:4n-6	0.04 ± 0.02	0.01 ± 0.03	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.03
20:5n-3	0.05 ± 0.02	0.02 ± 0.03	0.06 ± 0.01	0.05 ± 0.02	0.03 ± 0.03
22:5n-6	0.38 ± 0.04	0.38 ± 0.05	0.38 ± 0.07	0.36 ± 0.06	0.27 ± 0.06
22:6n-3	1.84 ± 0.13	1.78 ± 0.18	1.85 ± 0.22	1.80 ± 0.19	1.37 ± 0.24
Subtotal	10.1 ± 1.3 a	9.4 ± 1.3	9.6 ± 1.4	8.5 ± 1.0	7.64 ± 0.96 b
Total	18.1 ± 3.0 a	16.8 ± 2.6	16.8 ± 2.7	15.0 ± 2.0	13.0 ± 1.8 b

Means with different designations (a,b,c) are significantly different ($P<0.05$) from each other.

the BF_3 and the NaOMe catalyzed methods and with the AOCS method, significantly more branched chain FAME were present than with any other methods. Of all five procedures, the NaOMe catalyzed method produced the smallest concentration of total FAME. This likely occurs because basic catalysts such as that employed with the NaOMe method are unable to methylate any FFA that are present, resulting in lower total FAME concentrations. It should also be noted that while there were no significant differences among FAME concentrations of any acidic methods, the BF_3 method did produce the largest PUFA and total FAME concentrations. This is important because Christie (1989; 1993) has stated that the use of BF_3 leads to lowered PUFA yields.

The FAME data, expressed as percent of total (Table 3.6), also displayed some interesting variations. The AOCS method produced significantly higher ($P<0.001$) proportions of both branched chain and monounsaturated FAME than the other methods. This method also generated significantly lower ($P=0.008$) proportions of saturated FAME. These same trends were also evident in the absolute concentration data but the differences only became significant when expressed as proportions. These differences were due to differences in individual FAME proportions, specifically the presence of significantly more ($P<0.001$) 16:1n-9 and significantly less ($P<0.001$) 14:0 in the AOCS data. Desvilettes *et al* (1997) state that 16:1n-9 is a common freshwater bacterial fatty acid and this information, combined with the higher yield of branched chain fatty acids obtained with the AOCS method, suggests that this method may be particularly effective at esterifying bacterial lipids. Perhaps

Table 3.6 Variation in fatty acid proportions (% total fatty acids) in cultures of *Isochrysis galbana* with differing derivatization procedures (mean ± standard deviation, n=4-5).

	Boron Trifluoride	Hydrochloric Acid	Sulphuric Acid	AOCS	Sodium Methoxide
Branched					
i-15:0	0.33 ± 0.19	0 ± 0	0 ± 0	0 ± 0	0.11 ± 0.13
ai-15:0	0.07 ± 0.16	0.23 ± 0.37	0 ± 0	0.76 ± 0.10	0 ± 0
ai-16:0	0.48 ± 0.28	0.55 ± 0.13	0 ± 0	1.37 ± 0.18	0 ± 0
Subtotal	0.87 ± 0.31 a	0.78 ± 0.47 a	0 ± 0 c	2.13 ± 0.19 b	0.11 ± 0.13 c
Saturated					
14:0	17.0 ± 1.2 a	17.69 ± 0.37 a	17.54 ± 0.70 a	13.20 ± 0.38 b	15.89 ± 0.82 a
15:0	0.32 ± 0.05	0.17 ± 0.25	0.34 ± 0.03	0.29 ± 0.01	0.80 ± 0.21
16:0	8.17 ± 0.73	7.81 ± 0.62	8.13 ± 0.69	7.32 ± 0.52	7.91 ± 0.73
18:0	0.15 ± 0.15	0.30 ± 0.16	0.17 ± 0.10	0.22 ± 0.03	0 ± 0
Subtotal	25.7 ± 1.9 a	25.97 ± 0.86 a	26.2 ± 1.1 a	21.04 ± 0.84 b	24.6 ± 1.6 a
Monounsaturated					
16:1n-9	0.82 ± 0.49 a	0 ± 0 a	0 ± 0 a	3.66 ± 0.79 b	0 ± 0 a
16:1n-7	6.12 ± 0.39	6.09 ± 0.54	5.91 ± 0.23	5.54 ± 0.32	5.79 ± 0.27
18:1n-9	8.76 ± 0.21	9.13 ± 0.10	9.20 ± 0.14	9.81 ± 0.37	8.6 ± 1.2
18:1n-7	1.72 ± 0.13	1.91 ± 0.10	1.69 ± 0.10	1.06 ± 0.30	1.83 ± 0.89
Subtotal	17.42 ± 0.90 a	17.12 ± 0.49 a	16.80 ± 0.12 a	20.07 ± 0.63 b	16.19 ± 0.66 a
Polyunsaturated					
16:2n-4	1.29 ± 0.05	1.55 ± 0.14	1.23 ± 0.06	0.99 ± 0.30	1.18 ± 0.24
16:3n-4	0.34 ± 0.03	0.56 ± 0.11	0.35 ± 0.02	0.31 ± 0.03	0.85 ± 0.52
16:4n-1	0.40 ± 0.03	0.22 ± 0.20	0.44 ± 0.01	0.36 ± 0.02	0.21 ± 0.20
18:2n-6	20.09 ± 0.28	20.14 ± 0.24	20.15 ± 0.23	19.72 ± 0.14	21.00 ± 0.29
18:3n-6	1.88 ± 0.04	1.88 ± 0.07	1.85 ± 0.06	1.82 ± 0.04	2.11 ± 0.09
18:3n-3	9.06 ± 0.42	9.01 ± 0.32	9.15 ± 0.34	9.05 ± 0.24	9.32 ± 0.44
18:4n-3	9.99 ± 0.42	9.64 ± 0.24	9.87 ± 0.14	9.50 ± 0.13	10.84 ± 0.38
20:2n-6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.49 ± 0.33
20:4n-6	0.22 ± 0.12	0.07 ± 0.16	0.33 ± 0.02	0.30 ± 0.02	0.17 ± 0.24
20:5n-3	0.29 ± 0.07	0.12 ± 0.17	0.33 ± 0.03	0.32 ± 0.09	0.20 ± 0.20
22:5n-6	2.14 ± 0.18	2.28 ± 0.12	2.24 ± 0.08	2.41 ± 0.11	2.09 ± 0.36
22:6n-3	10.3 ± 1.1	10.67 ± 0.73	11.09 ± 0.70	11.98 ± 0.46	10.6 ± 1.7
Subtotal	56.0 ± 1.8 a	56.1 ± 1.5	57.0 ± 1.2	56.76 ± 0.79	59.1 ± 2.2 b
Total	100	100	100	100	100

Means with different designations (a,b,c) are significantly different ($P<0.05$) from each other.

these bacterial fatty acids are located in a more protected *sn*-2 position within the phospholipid so that a more vigorous reaction is necessary to transesterify those fatty acids. However, smaller proportions of the monounsaturates 16:1n-7 and 18:1n-7 were also present in the AOCS data. It is also possible that the conditions employed with that method were harsh enough to cause migration of the double bond in those acids and the only other technique producing 16:1n-9 was the BF_3 method. The AOCS method also employed BF_3 to esterify any FFA that were present, suggesting that that reagent may actually be capable of causing isomerization. In any case, there were few significant differences in proportions among the other four methods, and if data are to be reported only as percent of total, the most convenient method may be employed.

3.3.4 Removal of Hydrocarbon Contamination

Using the procedure described here, it was possible to remove hydrocarbon contamination and recover FAME in the same proportions as were present before clean-up. However, the proportion of diethyl ether in the solvent used to elute the FAME is critical. Table 3.7 contains ratios of peak areas before and after hydrocarbon removal in two samples for a variety of FAME relative to the same ratio for 16:0. Values near 1.0 represent close to 100% recovery relative to 16:0 while values much less than 1.0 represent losses of the FAME. Initial attempts to elute FAME with 99:1 hexane:diethyl ether did recover methyl esters but proportions of PUFA were significantly reduced (Table 3.8). Clearly, recovery was a function of double bond number rather than chain length with ratios of peak areas before

Table 3.7 Recoveries of individual FAME after hydrocarbon removal using different elution solvents, expressed as normalized ratios of peak areas before and after clean-up.

	Algal Sample 1 99:1 hexane:diethyl ether	Algal Sample 3 80:20 hexane:diethyl ether
Branched		
i-15:0	0.99	—
ai-15:0	0.00	1.06
ai-16:0	—	1.01
Saturated		
14:0	1.05	0.99
16:0	1.00	1.00
22:0	1.02	—
Monounsaturated		
16:1n-9	0.95	1.06
16:1n-7	0.96	1.00
17:1	0.70	—
18:1n-9	0.97	—
18:1n-7	0.96	1.03
Polyunsaturated		
16:2n-6	0.62	0.98
16:3n-4	—	0.95
18:2n-6	0.85	1.08
18:3n-3	0.55	1.02
18:4n-3	0.23	1.02
20:5n-3	—	1.03
22:4n-6	0.37	—
22:6n-3	0.15	1.00

* Peak areas were normalized to the peak area of 16:0 by the following equation:

$$\text{Normalized Area} = \frac{\dfrac{\text{Fatty Acid Area}_{\text{after}}}{\text{Fatty Acid Area}_{\text{before}}}}{\dfrac{\text{16:0 Area}_{\text{after}}}{\text{16:0 Area}_{\text{before}}}}$$

Table 3.8 Recoveries of FAME after hydrocarbon removal using different elution solvents.

	Average Normalized * Peak Area				
	Saturates	Monoenes	Polyenes	Total	Average Coefficient of Variation (%)
99:1 hexane:diethyl ether					
Algal Sample 1	1.02 ± 0.03	0.91 ± 0.12	0.46 ± 0.26	0.76 ± 0.31	40.4
Algal Sample 2	0.97 ± 0.05	0.97 ± 0.02	0.71 ± 0.19	0.85 ± 0.18	21.6
80:20 hexane:diethyl ether					
Algal Sample 3	1.10 ± 0.10	1.04 ± 0.02	0.99 ± 0.06	1.04 ± 0.08	7.8
Algal Sample 4	0.99 ± 0.05	0.97 ± 0.03	0.85 ± 0.06	0.93 ± 0.08	8.7
Algal Sample 5	1.01 ± 0.03	1.03 ± 0.03	1.00 ± 0.03	1.01 ± 0.03	3.0
Algal Sample 6	1.04 ± 0.05	1.00 ± 0.06	0.99 ± 0.02	1.01 ± 0.05	4.8

* Peak area were normalized to the area of 16:0 as in Table 3.7.

and after clean-up decreasing with degree of unsaturation, while no change was apparent in the ratio with increases in chain length. Mixtures of 90:10 hexane:diethyl ether were also not of sufficient polarity to recover all PUFA. The current method employing 80:20 hexane:diethyl ether yielded ratios of peak areas before and after clean-up that were very near unity with coefficients of variation less than 10% for all FAME, from saturates to polyunsaturates (Table 3.8). These data are conclusive evidence that FAME were being recovered after clean-up in equivalent proportions to the original FAME levels. This information also safely allows the addition of a FAME internal standard, if necessary, before hydrocarbon removal. Chromatograms before and after hydrocarbon removal are shown in Figure 3.4, displaying the effectiveness of this clean-up step.

3.3.5 Picolinyl Ester Formation

Reports in the recent literature (Christie, 1989; Harvey, 1992; Christie, 1998) describing the formation of picolinyl derivatives of fatty acids all begin with the reaction of free fatty acids with some reagent to form a more reactive compound, typically an acid chloride or anhydride. This requires a previous hydrolysis step of either the original lipid extract or the FAME derivatives. Hydrolysis involves additional sample handling steps where contamination may be introduced or sample loss may occur. Typical saponification procedures (Christie, 1989; Hamilton *et al*, 1992) also recommend that the lipid extract be refluxed, a procedure that will expose lipids to air and heat and may result in the oxidation of some PUFA. To avoid these hazards of saponification, a single-step transesterification

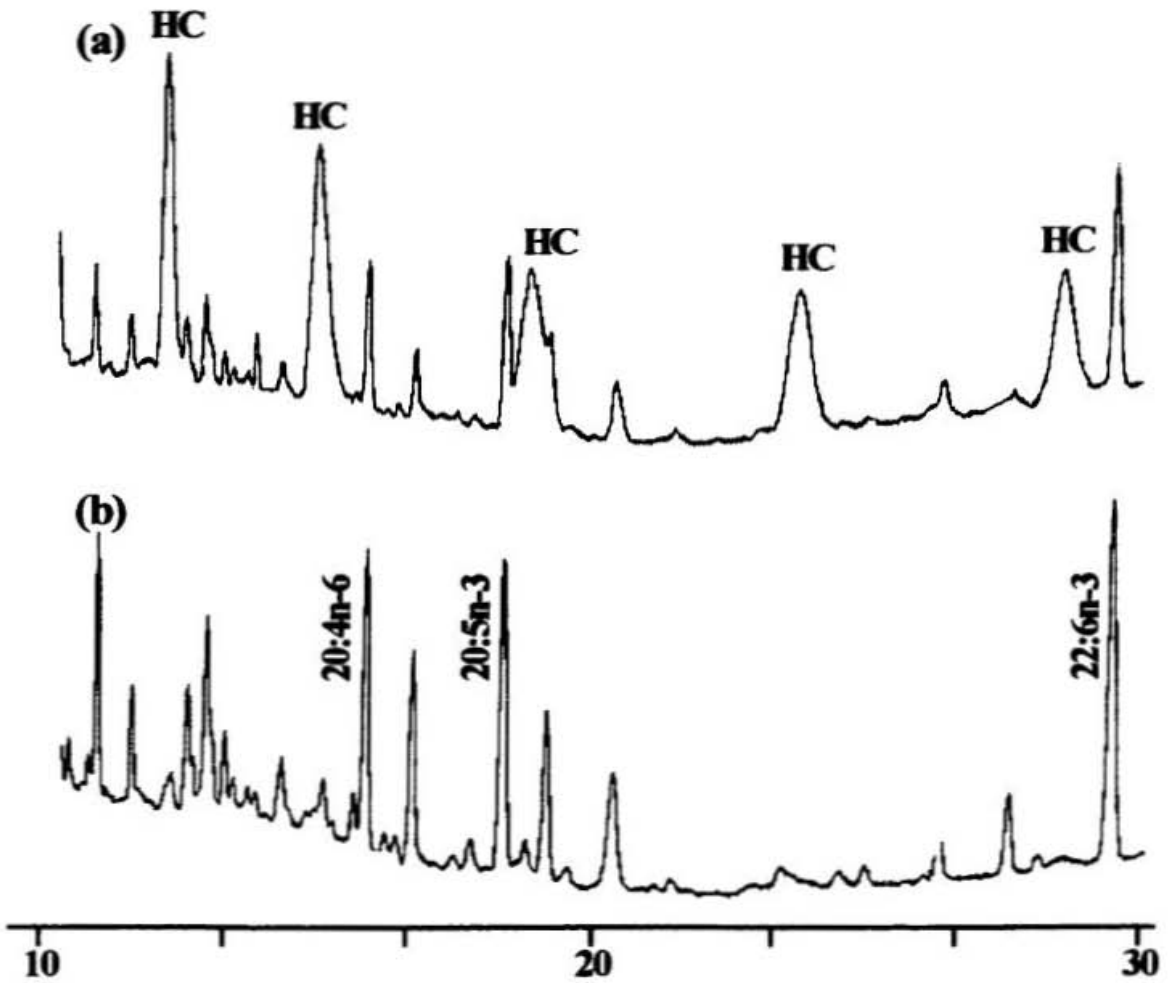
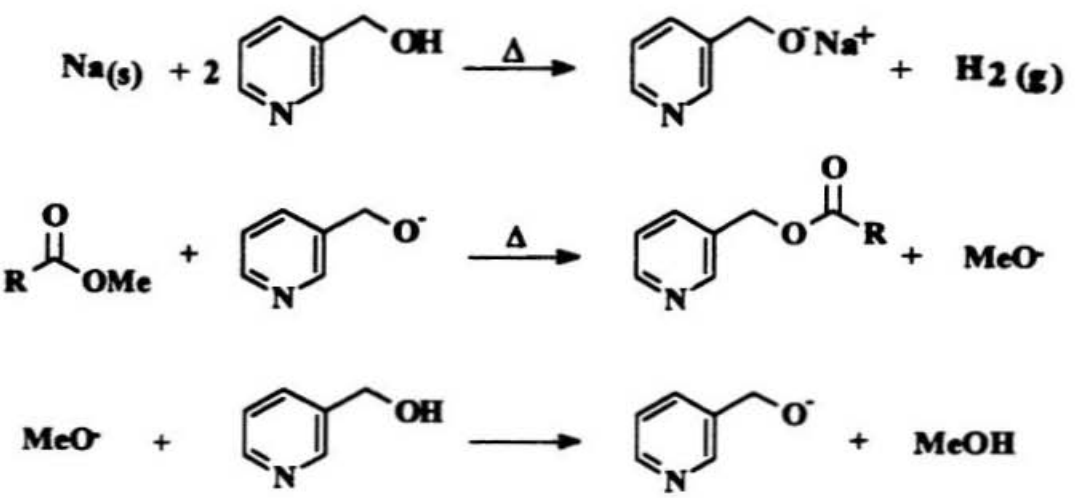


Figure 3.4 GC-FID chromatograms of FAME before (a) and after (b) removal of hydrocarbon contamination in a bivalve sample.

procedure for the formation of picolinyl esters was developed.

The procedure described here is a simple base-catalyzed transesterification similar to that employed in the NaOMe methylation of acyl lipids. The alkoxide ion of 3-(hydroxymethyl)pyridine was formed by dissolving elemental sodium in 3-(hydroxymethyl)pyridine. The alkoxide ion was then reacted with either the acyl lipids or methyl esters to form picolinyl esters. Methoxide ions generated as a product reacted with the excess alcohol to regenerate the alkoxide ion of 3-(hydroxymethyl)pyridine and methanol. In a study of the kinetics of the formation of butyl esters by transesterification from methyl esters, Roelofsen *et al* (1970) used molecular sieves to remove the methanol that was generated. Here, molecular sieve (3Å) was also employed to remove methanol and drive the reaction to completion. Heating for 1 hour at 80 °C was also found to be necessary. The procedure is summarized in the following 3 reactions:



This transesterification procedure was applied to the methyl esters of *M. edulis* tissue and examination of the total ion chromatogram (TIC) of the hexane-extractable reaction products reveals only picolinyl esters (Figure 3.5a). There was no evidence of unreacted methyl esters, suggesting complete conversion of methyl esters to picolinyl esters. For comparison, a second TIC of reaction products generated using hydrolysis followed by esterification (Christie, 1993) is also included (Figure 3.5b). While some picolinyl esters were formed, a variety of unreacted methyl esters were clearly present, indicating that incomplete hydrolysis was a problem. Undoubtedly, hydrolysis could be forced through the use of stronger base, higher reaction temperatures and longer reaction times, but preservation of original PUFA structures is questionable when using such harsh conditions. However, use of the transesterification procedure developed here produces quantitative conversion of FAME to picolinyl esters without compromising lipid structure.

A typical fragmentation pattern of the picolinyl ester of an unusual fatty acid is presented in Figure 3.6. This type of non-methylene interrupted fatty acid has been reported in bivalves (Joseph, 1982) but confirmation of its structure is always difficult because authentic standards do not exist, making a mass spectral identification necessary. Harvey (1992) has outlined mechanisms for the formation of various ions to be expected from a variety of picolinyl esters but he gives no information specifically applicable to fatty acids containing double bonds separated by more than 2 methylene groups. In Figure 3.6, a molecular ion at *m/z* 399 is obvious, allowing the fatty acid to be assigned a length of 20 carbon atoms with 2 double bonds. Prominent ions are also apparent at *m/z* 164

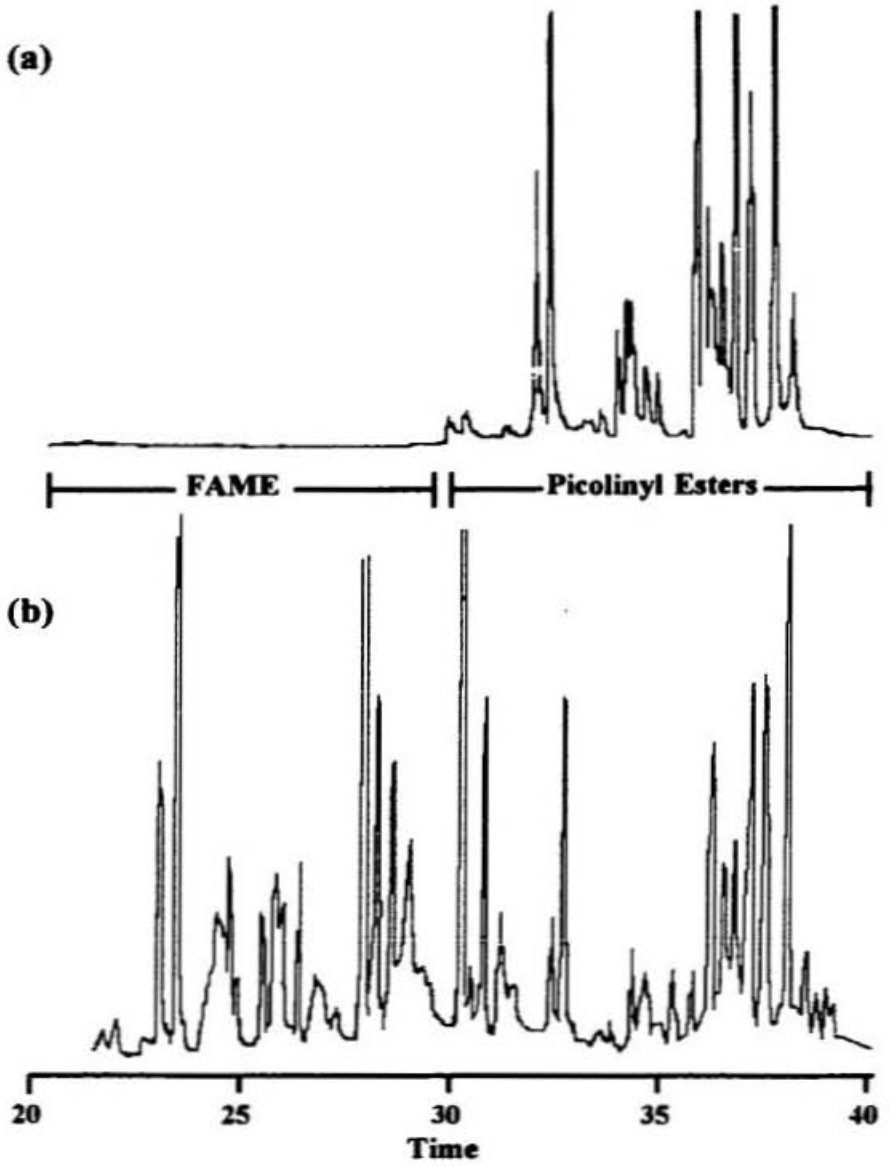


Figure 3.5 Total ion chromatograms of the hexane-extractable material recovered after formation of picolinyl esters. (a) Picolinyl esters formed formed by transesterification of FAME and (b) picolinyl esters formed by hydrolysis of FAME, followed by reaction with 3-(hydroxymethyl)pyridine.

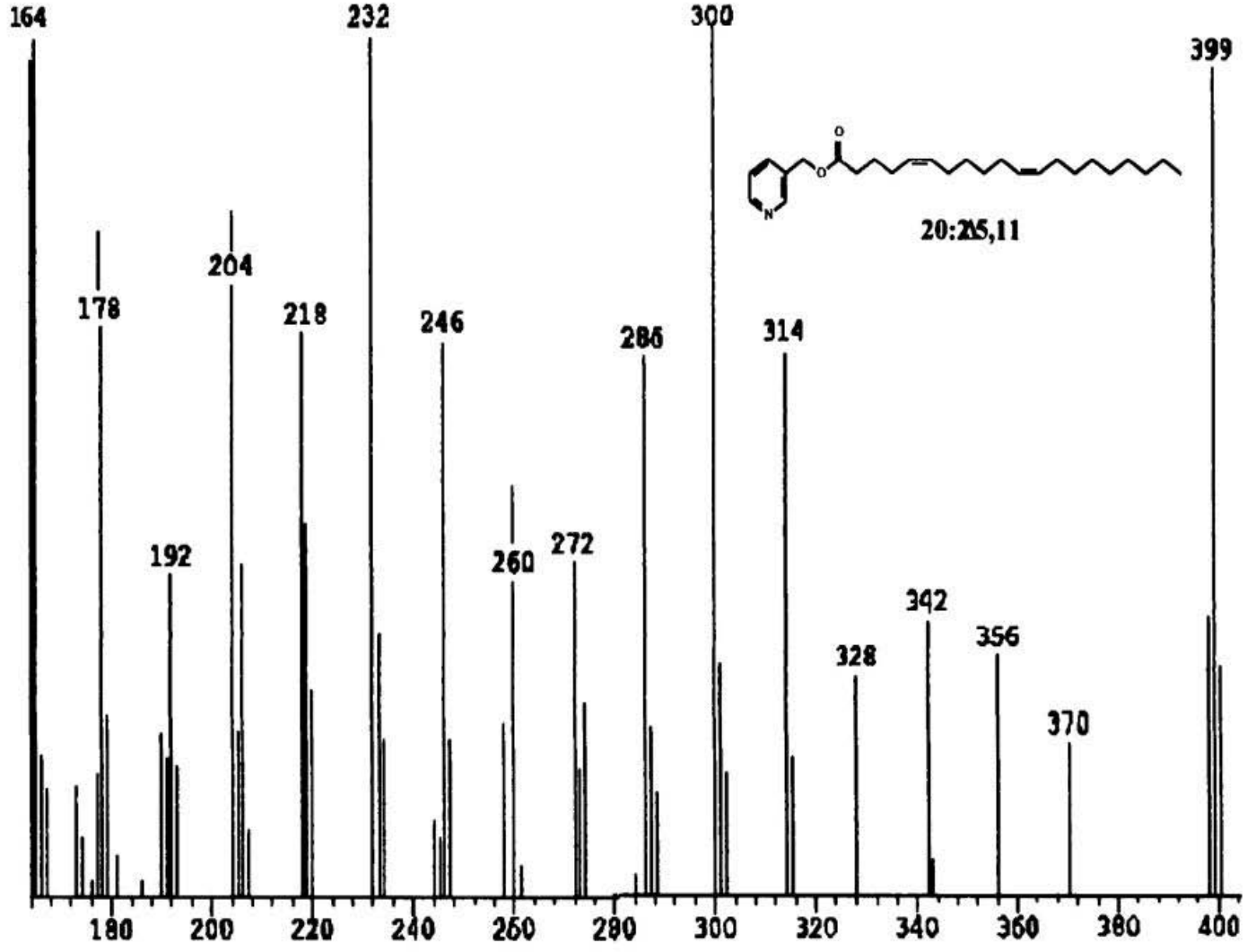
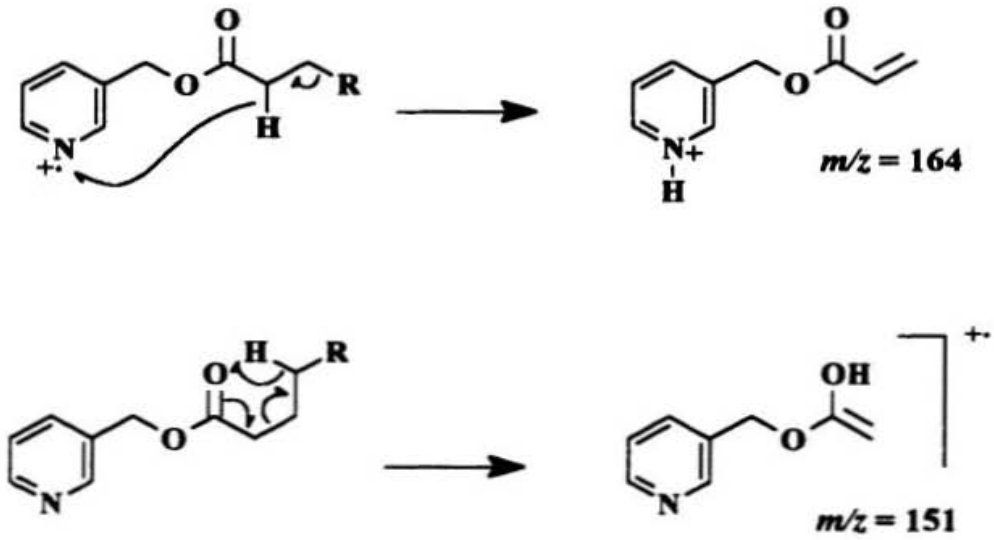
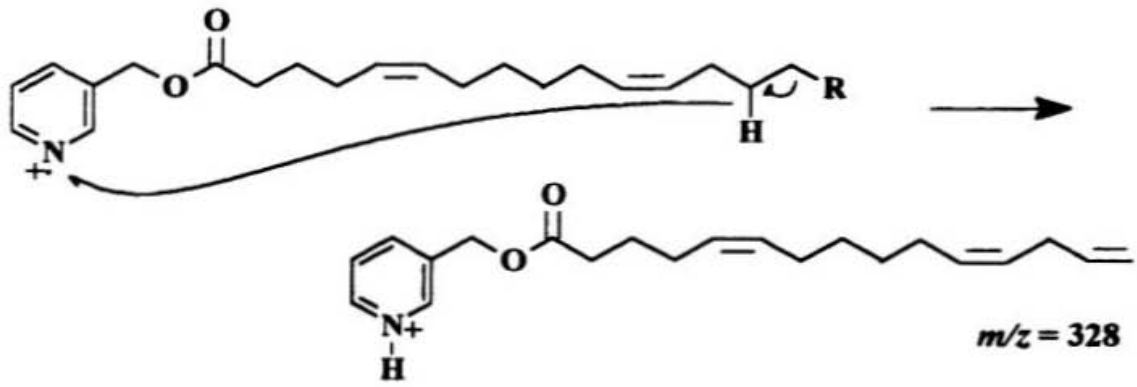


Figure 3.6. Mass spectrum of the picolinyl ester of 20:2Δ5,11.

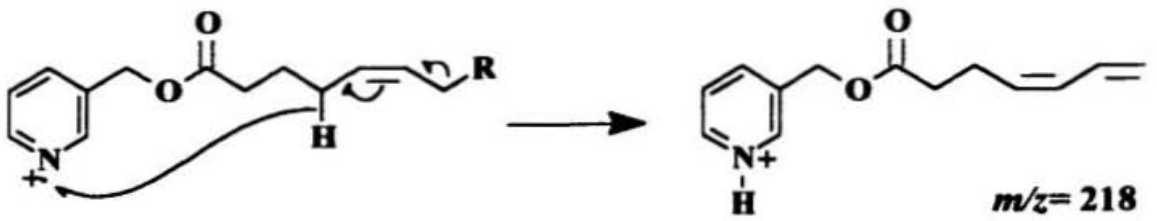
and 151 (not shown in Figure 3.6). Harvey (1992) describes the mechanisms for their formation as follows:



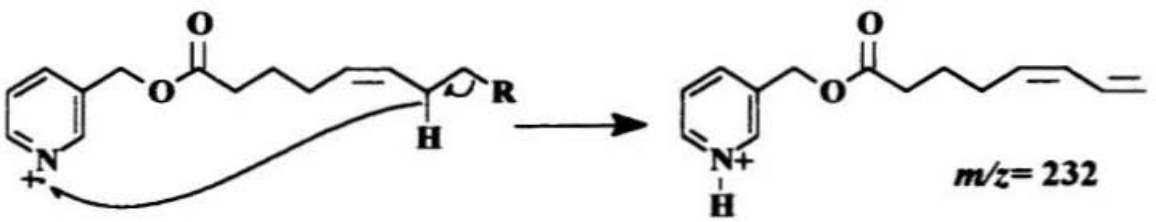
A series of diagnostic ions, such as those of *m/z* 328, 342, 356, and 370, is also generated by the following mechanism:



The 40 unit gap between *m/z* 178 and 218, and between 246 and 286, likely due to suppression of the previous reaction, suggests that the double bonds are located in that area. The bonds are fixed at the Δ5 and Δ11 positions by the presence of ions of *m/z* 218 and 300 generated by the following mechanism:



Ions at *m/z* 232 and 314 are also diagnostic of bond position due to the following:



Using these diagnostic ions, the identity of other NMID fatty acids including 20:2Δ5,13, 22:2Δ7,13 and 22:2Δ7,15 was confirmed in samples of *M. edulis*.

3.3.6 Estimation of Fatty Acid Concentrations using Acyl Lipid Data

Internal standards are not commonly employed in fatty acid analysis of marine samples because the fatty acid composition of these samples are very complex and an initial GC

analysis is necessary to ensure that the internal standard does not co-elute with any FAME. Use of internal standards may actually double the number of analyses required. Because of this, fatty acid data are generally reported as weight percent of total, especially in biological samples, giving no information about actual concentration. However, lipid class data are invariably expressed as concentration, making it possible to estimate FAME concentrations from acyl lipid data provided by TLC-FID. First, percent weight of total fatty acid data are converted to mole percent data. These mole percent data allow calculation of an average number of double bonds and carbon atoms in a particular sample. This, in turn, allows calculation of an average fatty acid molecular weight which may then be applied, with a series of calculations, to the acyl lipid data to determine a molar mass for each acyl lipid class. With that molar mass, the fatty acid contribution to mass for each acyl lipid class may be determined. These calculations are performed within a spread sheet and a table illustrating the results at each step is included in Appendix I. This calculation produces a very accurate estimate for lipid classes where the non-acyl portion of the molecule is known with certainty, such as with TAG, FFA and PL. Inaccuracy increases with lipid classes such as SE/WE and AMPL where the non-acyl portion of the lipid is not known. This is a particular problem with the AMPL group which contains non-acylated pigments along with glycolipids and MAG. Likely structures for the backbones can be proposed, but estimates can be expected to be less reliable as AMPL portions increase.

In Table 3.9, the non-acyl lipid structures used to calculate molar mass are shown. In algae, it was assumed (Parrish *et al*, 1994; Parrish *et al*, 1997) that approximately one third

Table 3.9 Non-acyl structures of acyl lipids used to estimate fatty acid methyl ester concentrations from acyl lipid data.

Steryl Esters	24-methylcholesta-5,22-dien-3β-yl ester
Wax Esters	C_{16} Hydrocarbon
Methyl Esters	As is
Triacylglycerols	Glycerol
Free Fatty Acids	Add CH_3
Diacylglycerols	Glycerol
Acetone-Mobile Polar Lipids	DGDG in algae, Cerebroside in animal tissues, SQDG in sediments
Phospholipids	Glycerol and Choline

of the AMPL peak consisted of DGDG. A similar assumption was made with the calculation for animal tissue except that a cerebroside structure was substituted. Steryl/wax esters (SE/WE) were determined as one peak and usually comprised a very small portion of total lipid (<5%). For ease of calculation, a 1:1 ratio of SE:WE was assumed with a C_{16} hydrocarbon chain in the WE portion and a 24-methylcholesta-5,22-dien-3β-yl ester in the SE portion (Wakeham and Frew, 1982).

To evaluate the accuracy of these estimates, three different marine samples (*Nannochloropsis* sp., *Mytelis edulis* and Trinity Bay sediments) were analysed for both lipid class concentration and FAME proportions, and FAME concentrations were estimated from those data. For comparison, FAME concentrations were also determined in the same samples through the use of an internal standard (23:0 FAME). The results of these comparisons, as well as concentrations of TAG, FFA and PL, are shown in Table 3.10. For both the algae and the animal tissue, estimated and actual values were quite close and, in fact, there was not a significant difference in the two values for the algae sample. The values for the animal tissue, however, were significantly different (P=0.002) with a slight overestimation produced by the calculation method. For both the algae and animal tissue samples, the calculation based on TLC-FID data produced a 10-18% overestimation as compared to the GC-FID determination. Interestingly, Parrish (1987) found that lipid values determined by TLC-FID were only 85% of gravimetric values. This suggests that, as methods become more specific (for example, from total lipids by gravimetry to TLC-FID determined lipids to GC-FID determined FAME) some portion of the lipids is either being lost or misidentified. Such losses would be expected

Table 3.10 Estimated and actual FAME concentrations in marine samples (mean ± standard deviation, n =3-6).

		Nannochloropsis µg/40 mL culture		*M. edullis* µg/mL extract		Sediment µg/g dry weight	
Lipids	TAG	63 ±	48	1420 ±	160	0 ±	0
	AMPL	318 ±	49	183 ±	24	792 ±	56
	PL	307 ±	66	435 ±	86	338 ±	35
FAME	Estimated	371 ±	95	2220 ±	210	481 ±	38
	Actual	333 ±	59	1870 ±	160	75 ±	5
% Discrepancy		11 ±	4	18 ±	1	540 ±	50

with increases in the amount of sample manipulation. It is also possible that these methods are incorporating a non-lipid contribution or a non-acyl lipid in the final concentrations.

An examination of the results of the comparison for sediment samples revealed a huge overestimation with the calculation methods that could not be attributed to instrumental error or losses due to handling, and suggested that some assumption within the calculations was incorrect. It is difficult to deduce likely structures for non-acyl portions of AMPL and PL in sediments because little is known about the composition of these classes. With these marine sediments, thought to be primarily composed of terrestrial plant material (see Chapter 4), it seemed reasonable to continue to assume that one-third of the AMPL peak was DGDG and that PL were primarily composed of phosphatidyl choline. However, with the huge overestimation, one or both of these assumptions is obviously in error.

To locate the source of this error, the FAME composition of the neutral, AMPL and PL fractions were determined and compared with the estimated FAME concentrations of the fractions (Table 3.11). Errors were evident in all three fractions, with a particularly alarming problem in the neutral fraction: the bulk of the acyl neutral lipids were FFA so a very similar value would be expected in the amount determined by internal standard. This suggests that the problem with this fraction may not involve an overestimation but, rather, losses of FAME during sample manipulation. Short chain fatty acids (< 12 carbon atoms) are quite volatile and may easily be lost when samples are concentrated by evaporation of solvent using a stream of nitrogen. When 8:0 and 23:0 free fatty acid standards were carried through the transesterification process, losses were negligible until the samples were concentrated.

Table 3.11 Estimated and actual FAME concentrations (μg g dry weight^{-1}) in fractionated sediment extracts (mean ± standard deviation, n = 3).

		Neutral		AMPL		Phospholipids	
FAME	Actual	9 ±	3	59 ±	5	7 ±	2
	Estimated using 1/3 AMPL as DGDG	75 ±	2	158 ±	12	248 ±	26
	% Discrepancy	730 ±	30	170 ±	8	3440 ±	40
	Estimated using 1/10 AMPL as DGDG	———		48 ±	3	———	
	% Discrepancy	———		19 ±	8	———	
	Estimated using 1/10 AMPL as SQDG	———		53 ±	4	———	
	% Discrepancy	———		10 ±	8	———	

After partial evaporation, the concentration of 8:0 was reduced by 31±9% as compared to 23:0. Because of the difficulty associated with accurate quantitation, these short chain fatty acids are rarely reported in the literature but at least one study (Perry *et al*, 1979) did report that fatty acids with chain lengths as short as C_9 comprised approximately 8% of total fatty acids in sediments. Short chain FAME are apparent in the GC traces of the sediments in the current study and it is likely that losses of these volatile FAME are responsible for the discrepancy in the results. It should also be noted that the presence of short chain fatty acids is not included in the calculated value of chain length. If they were present, their exclusion in the calculated method would also contribute to the overestimation in FAME concentration.

The presence of short chain fatty acids may also play a role in the discrepancies in the AMPL and PL fractions but to obtain a better understanding of the lipid composition of these classes and their contribution to FAME concentration, more detailed TLC separations designed to further separate AMPL and PL classes into individual lipids, were carried out (Figure 3.7). The AMPL fraction was composed predominantly of chlorophyll *a* and some other unidentified green pigment, possibly chlorophyll *b*, but small amounts of the two glycolipids, DGDG and SQDG, were also present, comprising, on average, about 10% of total AMPL. Without considering the different responses of chlorophyll *a* and glycolipids, and using a molar mass based in DGDG, that would result in 48 μg g dry weight^{-1} of glycolipid contributing to the FAME concentration (Table 3.11). However, the broad SQDG peak actually comprised the bulk of this 10%, so a molar mass based on its non-acyl structure was

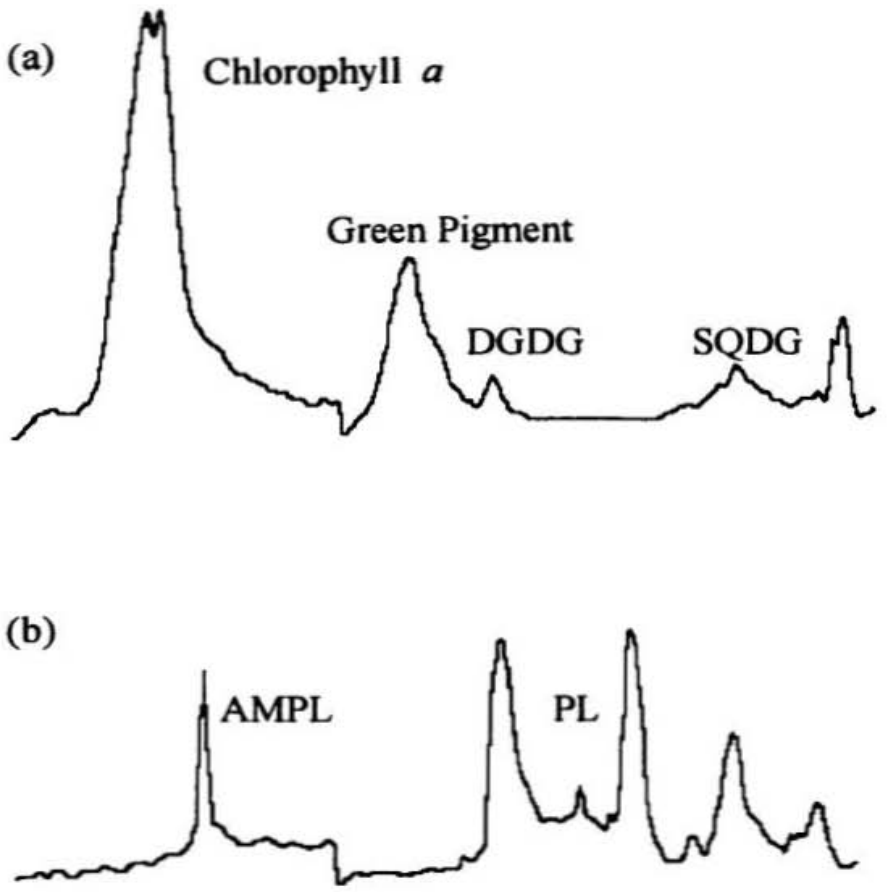


Figure 3.7 TLC-FID chromatogram of sediment samples. (a) Glycolipid separation of the AMPL fraction and (b) phospholipid separation of the PL fraction.

also used in the calculation and yielded a value of 53 ± 4 μg g^{-1}, closer still to that determined by internal standard. An interesting result was noted when comparing the chromatograms of the glycolipid separation of the total extract (Figure 3.8) and the AMPL fraction. Three additional peaks, two eluting between DGDG and SQDG and one between chlorophyll *a* and the green pigment, were evident in the unfractionated extract, indicating that some small loss of AMPL species did occur on the silica gel column. It is possible that the peaks eluting after DGDG may also be acyl lipids such as digalactosyl monoacylglycerol or monogalactosyl monoacylglycerol. These much more polar lipids are the expected hydrolysis products of DGDG and MGDG and could be generated naturally in the sediments or by sample handling.

While the TLC-FID separation gave some insight into the AMPL composition, it did little to resolve the questions concerning acyl composition of the PL fraction (Figure 3.8). The PL separation of the entire sediment extract yielded only two lipid peaks. The smaller peak of the two was visually identified as a blue pigment, probably a derivative of fucoxanthin (Jensen, 1964). Comparisons of retention times of PL standards were used in an attempt to identify the other, larger peak, but no identity was confirmed. However, from that exercise, it is now known that that peak is not composed of any of the PL commonly expected in marine samples, such as PC, phosphatidylethanolamine, phosphatidylglycerol or diphosphatidylglycerol. In any case, if the peak had been identified as one of those acyl PL, it would not explain the huge discrepancy in estimated and actual results. There is a leading shoulder on the unidentified peak that corresponds to the retention time of PC and it may be that the fatty acids are derived form this.

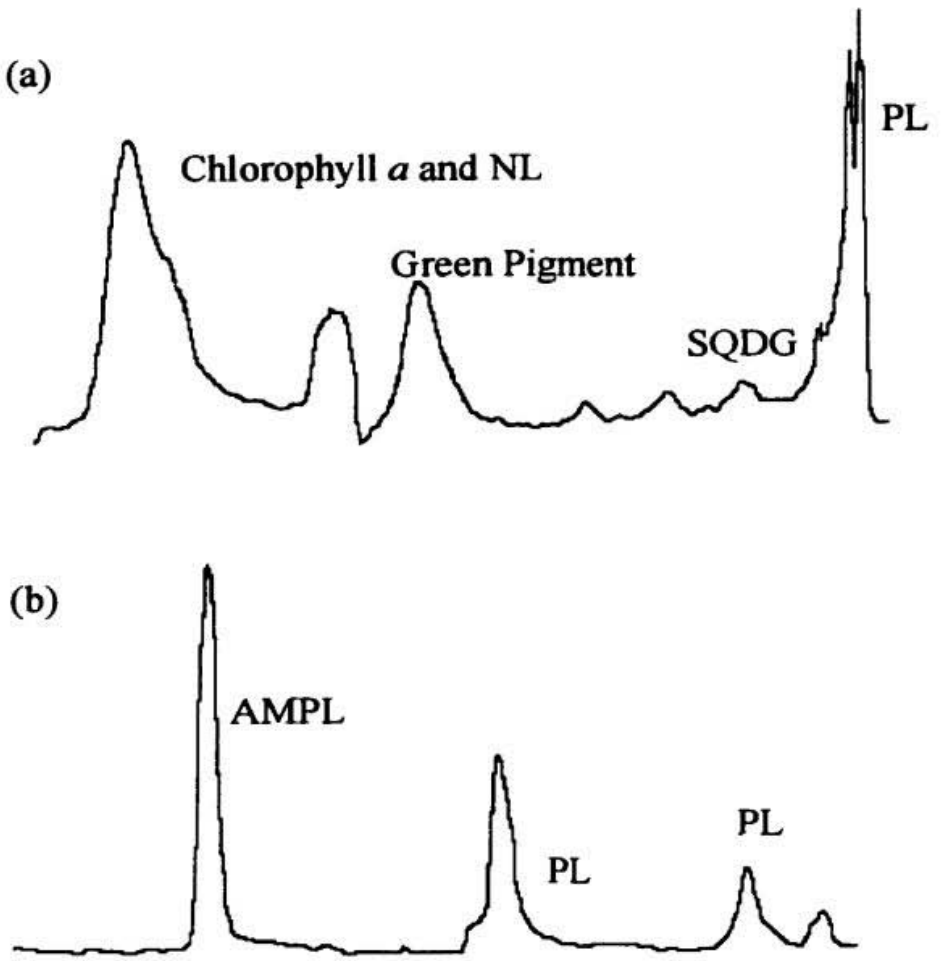


Figure 3.8 TLC-FID chromatogram of total lipid extracts of sediment samples: (a) Glycolipid separation and (b) phospholipid separation.

To further complicate matters, the chromatogram of only the PL fraction shows an additional PL peak (Figure 3.7), suggesting that some breakdown of the original unidentified compound had taken place to produce a slightly more polar compound. Initially, the presence of ether-linked PL were considered as a possible explanation of the observed results. These lipids are rather refractory because of the ether groups (Snyder, 1985) so their presence in sediments is reasonable and they are not acyl so they would not contribute to the FAME concentration. However, some breakdown was occurring on the silica gel column and it is very unlikely that the unreactive ethers could undergo any reaction on that column. An alkylacylglycerolipids, a combination of an acyl and an ether-linkage, is another possibility. This would generate some FAME, as observed, and such a compound can, in theory, undergo degradation resulting in loss of the acyl chain and production of a more polar compound. However, because of polarity, that type of structure would be expected to elute before, rather than after, PC, as observed here. Unfortunately, to date there is no information about the behaviour of ether-linked PL in the solvent systems employed in this study. It is possible that short-chain fatty acids may be affecting the chromatography, as well as the quantitative results. A conclusive identity could likely be obtained through high performance liquid chromatography with MS detection. However, until the identity of that peak is established, it will not be possible to estimate FAME concentrations with any confidence from lipid class data in sediments.

3.4 Conclusions

All aspects of methodology for the analysis of fatty acids in typical marine samples were critically evaluated and optimized. The speed and simplicity of the modified Folch *et al* (1957) extraction technique is preferred except with large samples of tissue where an initial homogenization step must be introduced. A column chromatography separation of the lipid extract can be performed to produce neutral, AMPL and PL fractions. However, only approximately 75% PL can be recovered from these silica gel columns and caution should be exercised when quantitative results are necessary from PL fractions. The extract can then be transesterified to form FAME with the most convenient acid-catalyzed procedure. In this laboratory, 10-14% BF_3 in methanol is routinely used as catalyst without any evidence of PUFA loss. A simple method to remove hydrocarbon contamination with column chromatography on silica gel can also be applied when necessary without selective loss of FAME on the column.

A new transesterification method for the formation of picolinyl esters for use in the mass spectral identification of fatty acids was developed. This new method is quantitative and offers the advantage of avoiding hydrolysis of the lipid extract and artefacts associated with that procedure. Finally, a simple series of calculations to estimate FAME concentration from acyl lipid data was developed and its application to several marine samples was discussed. Algae and animal tissue samples yielded estimates that were within 10-18% of actual FAME concentrations. However, the accuracy of the estimate decreased as knowledge of lipid structure decreased, with the most unreliable estimates being evident in the poorly

characterized sediment samples. This study did prompt the first detailed examination of AMPL and PL classes in sediments, revealing that pigments comprise the bulk of the AMPL and that an unusual non-acyl PL is present.

Chapter 4- FATTY ACID BIOGEOCHEMISTRY OF PLANKTON, SETTLING MATTER AND SEDIMENTS IN TRINITY BAY, NEWFOUNDLAND

4.1 Introduction

Marine biogeochemical studies of settling particulate matter are useful in determining carbon cycling and assessing the overall health of ecosystems. Primary production in coastal areas generates large amounts of organic matter of which a significant portion sinks through the water column and is ultimately preserved in sediments. Here the carbon cycle in a mid-latitude coastal environment with a subpolar temperature regime is investigated by examining the fatty acids of phytoplankton, settling particulate matter and sediments. Fatty acid biomarkers are used to determine sources of this organic matter and the extent that the composition and sources of this matter change as it sinks through the water column and is deposited on the sea floor. In addition, sources as determined by fatty acid biomarkers are compared to floristic determinations on the same samples.

Specific fatty acids have been used as biomarkers for almost two decades. Certain general markers, such as those for diatoms (Claustre *et al*, 1988-89) and bacteria (Volkman *et al*, 1980; Haddad *et al*, 1992; Wakeham and Beier, 1991; Harvey, 1994) are well accepted. However, markers for other sources of fatty acids are required. For example, the ratio of 16:0/24:0 has been used as an indicator of relative terrestrial plant contributions because of differing preservation tendencies (Gearing and Pocklington, 1990) but very little evidence to support its usefulness has been produced. Similarly, dinoflagellates are usually described

as containing elevated amounts of 22:6n-3 (Sargent *et al*, 1987; Harvey *et al*, 1988; Viso and Marty, 1993) but no appropriate indicator making use of this information exists. One of the primary objectives of this study was to investigate the use of established indicators for diatoms and bacteria and to explore the usefulness of new ones for terrestrial material and dinoflagellates.

As general diatom indicators, the ratios of 16:1/16:0 and ΣC16/ΣC18, as initially proposed by Claustre *et al* (1988-89), were used. In conjunction with this, Bodennec *et al* (1994) suggested that values of 16:1/16:0 greater than 1.6 can be interpreted as signalling the predominant presence of diatoms. Claustre *et al* (1988-89) interpreted an increase in values of both of these ratios as representative of increased proportions of diatoms. In addition to these two markers, the fatty acid, 16:4n-1, was also used to assess the importance of diatoms. This acid is commonly found in diatoms (Nichols *et al*, 1986; Viso and Marty, 1993) but is very rarely encountered in other phytoplankton, and it can be used as a general marker for diatoms.

To assess the importance of bacteria as a source of organic material and to determine the extent of bacterial degradation in samples, particularly in sediment traps and sediments, a bacterial indicator was required. Odd carbon-numbered and branched-chain fatty acids are commonly produced by both aerobic and anaerobic bacteria (Parkes and Taylor, 1983; Caudales and Wells, 1992; Harvey and Macko, 1997) and may be used as bacterial biomarkers (Volkman *et al*, 1980; Wakeham and Beier, 1991; Haddad *et al*, 1992; Harvey, 1994). Here, the sum of *iso*- and *anteiso*-branched chain acids and unbranched 15:0 and 17:0

was used to determine the presence of bacteria in these samples.

Sediment traps are commonly used to study settling particulate matter and have provided considerable information concerning fluxes and changes in composition of sinking lipid material (Wakeham *et al*, 1981; De Baar *et al*, 1983; Wakeham *et al*, 1984; Harvey and Johnston, 1995). However, there is some concern that degradation of material in these traps may occur during deployment due to microbial action. Lee *et al* (1992) investigated the effectiveness of various poisons and preservatives in sediment trap work and found that mercuric chloride, as well as other treatments, at sufficient concentrations, was capable of inhibiting bacterial growth. They also found large numbers of zooplankton in these poisoned traps which influenced the flux and composition of the settling particulate matter. In this study, comparisons of poisoned and non-poisoned traps at the same locations are made to determine the extent to which these treatments prevent bacterial growth at sub-zero temperatures.

A second issue in sediment trap studies involves the dissolution of particulate organic matter while in the trap. Körtzinger *et al* (1994) reported that dissolution of fatty acids from particulate material in sediment traps resulted in significant losses of particulate matter and underestimation of fluxes by 15 to 75%. The potential error that this could introduce into results was sufficient reason to more closely examine this process.

4.2 Experimental

4.2.1 Sample Collection

All marine samples were collected in Trinity Bay which is a large, fjord-like bay on the east coast of Newfoundland (Figure 4.1). Net-tow, sediment trap and sediment samples were taken at various stations (Table 4.1). During the spring bloom of 1996, 12 vertical net-tows (20 μm) were taken at St- 7, 9 and 11. Tows were taken from 100 m to the surface and plankton captured in the cod end were stored in bottles on ice. Lipid samples were taken by filtering known proportions of the resuspended plankton onto pre-combusted GF/C glass-fibre filters. Filters were then placed in chloroform and stored under nitrogen in the dark at -20 °C.

Sediment trap moorings were deployed at St-7 (offshore) and St-9 (nearshore) for various periods of time in 1995, 1996 and 1997 (Table 4.1). Traps were deployed at depths of 50, 75 and 100 m below the surface with four collection cylinders at each depth. One litre of 40 ppt NaCl solution was added to each trap and the remainder of each was filled with seawater before deployment. In May 1997, $HgCl_2$ was added as a poison to this NaCl solution in one of every four sample cylinders at a concentration of 5 g L^{-1}. On recovery, particles in the trap were allowed to settle for 30 minutes before draining off most of the water in the trap. The remaining water containing the particles was then collected in bottles. As with the plankton samples, the particles were resuspended and filtered on GF/C glass-fibre filters. In May 1997, instead of discarding the filtrate, the water was collected to determine dissolved lipids.

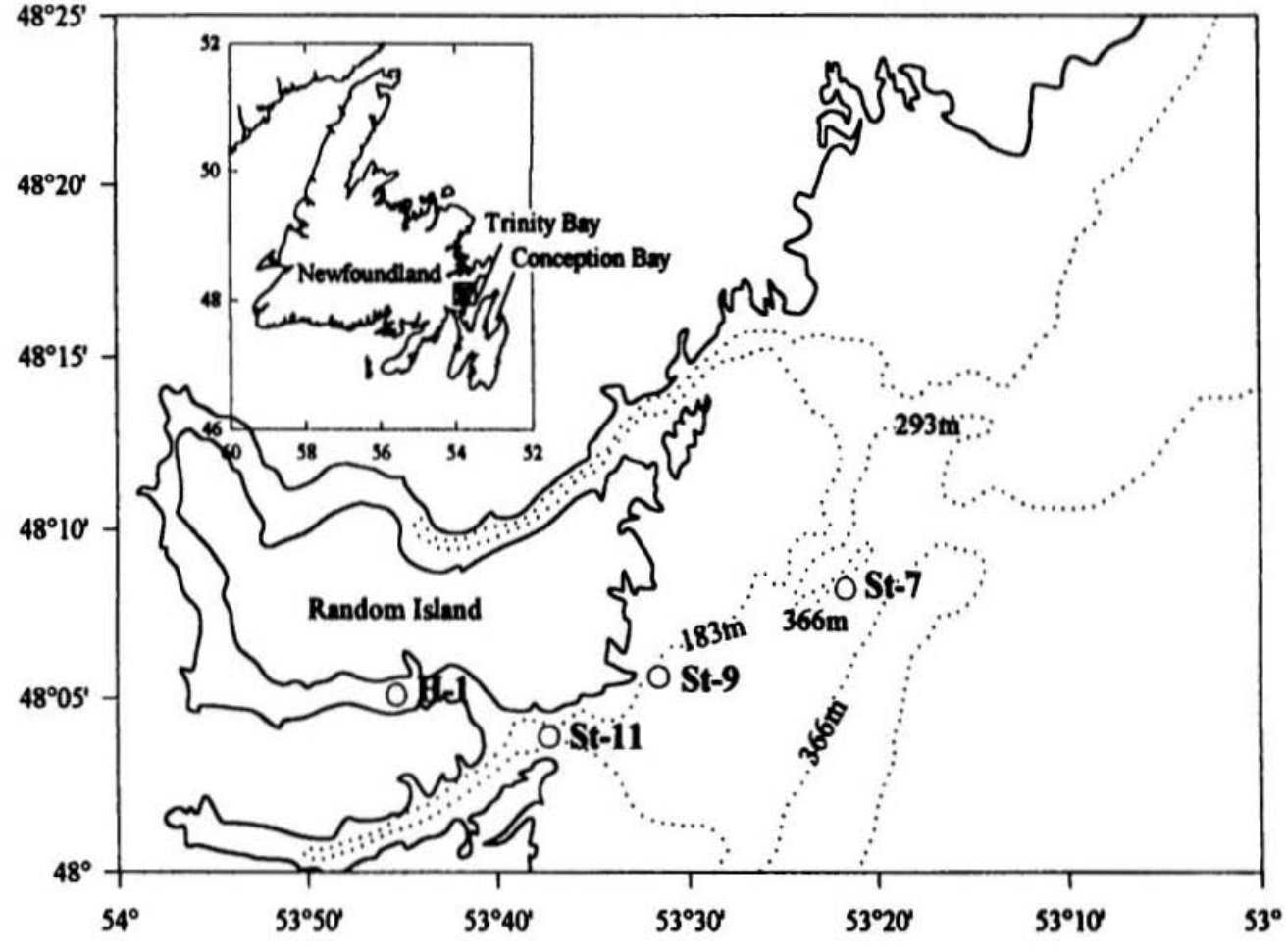


Figure 4.1 Locations of sampling stations in Trinity Bay.

Table 4.1 Locations, depths and types of samples collected in Trinity Bay.

Station	Latitude (°N)	Longitude (°W)	Depth (m)	Dates Sampled		
				Net-tows (0-100 m)	Sediment traps (50-100 m)	Sediment sampling
St-7	48 08.2	53 22.0	363	Mar - June 96	Jun - Jul 94 Jul 94 - Apr 95 May 97	30 cm core
St-9	48 05.7	53 31.7	224	Mar - June 96	Jun - Jul 94 Jul - Oct 94 Apr 95, May 97	-
St-11	48 03.8	53 37.8	314	Mar - Jun 96	-	-

A sediment core was collected by Jerry Pulchan at the offshore site (St-7) with a 30 cm box-corer. Samples were stored on ice and frozen on returning to land. Before extraction, sediments were dried in the dark at 30 °C and homogenized with a mortar and pestle by Yvette Favaro.

4.2.2 Extraction, Derivatization and Analysis

The particulate matter from net-tows and sediment traps collected on GF/C filters were extracted in chloroform-methanol using the modified Folch procedure as described in Section 3.2.3.1. The dissolved lipids in the filtrates from trap samples collected in 1997 were extracted with 40 mL of chloroform (20:1 water:chloroform) in a 1 L separatory funnel. The sediments were extracted using Soxhlet apparatus with dichloromethane:methanol (9:1) (Section 3.2.3.3). Fatty acid methyl esters were formed for all samples as described in Section 3.2.6.1 and were determined by GC-FID (Section 3.2.10.1).

4.2.3 Floristic Analysis

Subsamples of phytoplankton and trap material were preserved in Lugol's iodine and 10% buffered formaldehyde for microscopic analyses. From a well mixed sample, 200 µL were removed for microscopy by Denis Short and resuspended in approximately 2 mL of distilled water. An ocular micrometer and appropriate geometric shapes were used to estimate biovolume.

4.3 Results and Discussion

4.3.1 Description of Fatty Acid Markers

To assess the importance of terrestrial material entering this marine system through rivers and streams, a marker that was discernable in normal fatty acid analyses was required. Hence, the fatty acid composition of various samples of potential terrestrial input was examined. Terrestrial plants growing in or near streams in the Random Island watershed emptying into Trinity Bay were sampled and included *Equisetum* (a reed commonly known as horse tail), *Carex* (a grass) and *Juncus* (a rush). Samples of pollen that could be transported by wind into the ocean were analysed, and net-tow samples were taken near station H-1 in a river flowing directly into the Northwest Arm, off Trinity Bay. Terrestrial plants generally contain large amounts of 18:2n-6 and 18:3n-3 (Ackman, 1986) and the fatty acid analyses of the plant and pollen samples agreed with this (Table 4.2). The riverine samples contained much less of the two acids but the amounts of 18:2n-6 and 18:3n-3 still exceeded that of any marine samples examined in this study. In addition, Parrish *et al* (1995) examined the fatty acids in particulate matter in a shallow cove where terrestrial inputs were expected to be large and found amounts of 18:2n-6 and 18:3n-3 exceeding 4.7% of total fatty acids. These data suggested that the sum of 18:2n-6 and 18:3n-3 could be used as an indicator for the relative importance of terrestrial material. By examining marine net-tow data, which was assumed to contain little or no terrestrial material, an arbitrary threshold of 2.5% was assigned to this indicator. In this way, samples with values above this may be considered to have terrestrial material as a significant source of organic matter. Preliminary studies with

Table 4.2 Proportions (% total fatty acids) of 18:2n-6 and 18:3n-3 in terrestrial material (mean ± standard deviation, n=1-3).

	18:2n-6	18:3n-3
Equiseteum	13.97 ± 0.35	36.79 ± 0.69
Carex	11.3 ± 1.9	20.4 ± 4.0
Juncus	5.06 ± 0.64	4.10 ± 0.15
Pine Pollen	16.77	0.95
River Plankton	1.93 ± 0.20	1.86 ± 0.26

compound specific isotope analysis offer support for this marker as the analyses showed that 18:2n-6 and 18:3n-3 in the riverine samples were the most depleted of all fatty acids with $\delta^{13}C$ values of -33.4 and -33.6‰, respectively. The $\delta^{13}C$ values of these terrestrial fatty acids are discussed further in Section 6.3.4. In addition, a recent study (Napolitano *et al*, 1997) uses 18:2n-6 as a terrestrial marker.

A suitable indicator to distinguish the importance of dinoflagellates versus diatoms was also required. The acid 22:6n-3 alone fails as a marker for dinoflagellates, as many classes of phytoplankton also produce significant amounts of this acid. Several reports of culture samples (Volkman *et al*, 1989; Viso and Marty, 1993) have shown that diatoms generally produce far more 20:5n-3 than 22:6n-3, while dinoflagellates contain elevated amounts of 22:6n-3 and less 20:5n-3. Incorporating these two acids in a ratio, 22:6n-3/20:5n-3, should generate a useful indicator. From the culture data, values near or larger than unity would signal dinoflagellates as the predominant species, while values far less than one would indicate the presence of diatoms. Bodennec *et al* (1994) have also proposed the inverse of this ratio, 20:5n-3/22:6n-3, as an indicator for diatoms, *Thalassiosira pseudonana* specifically. The biomarkers used in this chapter and in Chapter 6 are summarized in Table 4.3.

4.3.2 Plankton

In late March, the offshore samples were dominated by centric diatoms, particularly *Thalassiosira* sp. and *Chaetoceros* sp. The pennate diatoms, *Nitzschia* sp. and *Navicula* sp., were also present as a small proportion of total diatoms. Surprisingly, approximately 20% of

Table 4.3 Summary of biomarkers used in Chapters 4 and 6. Where specific levels are not indicated, increasing biomarker values indicate the increasing importance of the particular source relative to other samples.

Biomarker	Source
16:1/16:0	>1.6; diatoms
ΣC16/ΣC18	diatoms
16:4n-1	diatoms
22:6n-3/20:5n-3	dinoflagellates
18:5n-3	dinoflagellates
18:2n-6 + 18:3n-3	>2.5; terrestrial plants
20:0 + 22:0 + 24:0	terrestrial plants
Σodd carbon numbered + branched chain fatty acids	bacteria

the algal cells were the armoured dinoflagellates, *Ceratium tripos*, *Dinophysis* sp. and *Scrippsiella* sp. In Newfoundland, these dinoflagellates usually only appear in midsummer, after the spring diatom bloom (Parrish *et al*, 1995). In early April, increasing numbers of centric diatoms, particularly *Chaetoceros* sp. and *Leptocylindrus danicus*, were found and an obvious *Chaetoceros* sp. bloom was present in early May samples. Small numbers of the centric diatoms, *Thalassiosira* sp., *Leptocylindrus danicus* and *Coscinodiscus* sp., were also encountered during this bloom. Finally, offshore in late June, the diatom bloom had subsided and the dinoflagellate *Ceratium tripos* became the dominate species. Copepod nauplii were also present to varying degrees in all net-tow samples examined.

The average concentrations of fatty acids in net-tow samples at all three sampling sites for each date are given in Table 4.4. A large fatty acid concentration was found in May, reflecting the contribution of the diatom algal cells to organic matter in the water column. The greater concentration in June after the bloom may have been due to the presence of the dinoflagellate *Ceratium tripos* whose individual cells are quite large (approximately 2200 μm^3) and comprised about 85% of total cells counted at this time. A similar explanation may apply to the March samples which were surprisingly elevated in fatty acid concentration. The concentrations of PUFA mirrored those of total fatty acids, likely for similar reasons.

Percent composition of individual fatty acids in the net-tows also reflected the changing phytoplankton species composition. Few significant differences were found among net-tows collected at different sites on the same sampling date so data from the offshore site (St-7) are presented (Table 4.4 and Figure 4.2). The fatty acid composition of net-tows taken

Table 4.4 Fatty acids in net-tows collected during the spring of 1996 (mean ± standard deviation, n=3). Total fatty acid and PUFA concentration (mg g dry weight^{-1}) are averages of stations St-7, St-9 and St-11. Fatty acid proportions (% total fatty acids) are averages of 3 subsamples of net-tows from the offshore station (St-7).

Fatty Acid	20 March 1996		03 April 1996		07 May 1996		28 June 1996	
Total Conc	17.8 ±	5.9	2.6 ±	1.2	31.5 ±	9.6	41 ±	17
PUFA Conc	7.4 ±	3.0	0.80 ±	0.40	12.8 ±	4.6	22 ±	10
Branched								
i-15:0	0.24 ±	0.03	0.19 ±	0.04	0.22 ±	0.06	0.31 ±	0.06
ai-15:0	0.20 ±	0.04	0.32 ±	0.06	0.49 ±	0.10	0.31 ±	0.04
i-16:0	0.16 ±	0.06	0.08 ±	0.05	0.07 ±	0.03	0.20 ±	0.01
Subtotal	0.61 ±	0.11	0.59 ±	0.07	0.78 ±	0.18	0.82 ±	0.10
Saturated								
14:0	8.4 ±	1.3	10.05 ±	0.59	12.2 ±	1.5	6.73 ±	0.54
15:0	0.43 ±	0.03	0.49 ±	0.03	0.60 ±	0.23	0.54 ±	0.13
16:0	9.83 ±	0.53	12.61 ±	0.81	10.9 ±	1.2	13.2 ±	1.0
17:0	0.16 ±	0.06	0.21 ±	0.06	0.09 ±	0.06	0.47 ±	0.12
18:0	0.52 ±	0.17	1.71 ±	0.04	0.79 ±	0.07	1.39 ±	0.17
20:0	0.27 ±	0.37	0 ±	0	0.26 ±	0.07	0.02 ±	0.04
22:0	0 ±	0	0.30 ±	0.30	0.07 ±	0.03	0 ±	0
Subtotal	19.65 ±	0.92	25.4 ±	1.7	25.0 ±	3.0	22.4 ±	1.3
Monounsaturated								
16:1n-7	9.93 ±	0.68	9.0 ±	2.5	21.5 ±	1.6	4.7 ±	1.1
16:1n-5	0.25 ±	0.19	0.44 ±	0.48	0.06 ±	0.10	0.53 ±	0.46
18:1n-9	10.4 ±	1.6	6.5 ±	1.1	4.4 ±	2.6	6.84 ±	0.82
18:1n-7	0 ±	0	0 ±	0	0.36 ±	0.63	0 ±	0
18:1n-5	1.20 ±	0.22	1.12 ±	0.51	0.41 ±	0.05	1.24 ±	0.23
20:1n-11	0.89 ±	0.26	2.27 ±	0.48	0.32 ±	0.10	2.36 ±	0.58
20:1n-9	0.8 ±	1.3	0 ±	0	0 ±	0	0 ±	0
20:1n-7	4.5 ±	2.0	3.3 ±	1.3	0.50 ±	0.20	1.09 ±	0.41
22:1n-11	0 ±	0	0.24 ±	0.42	0.12 ±	0.06	0.05 ±	0.03
22:1n-9	4.84 ±	0.72	1.98 ±	0.40	0.15 ±	0.23	0.94 ±	0.92
24:1	0.68 ±	0.17	1.75 ±	0.39	0.07 ±	0.07	0.22 ±	0.09
Subtotal	33.5 ±	2.5	26.6 ±	5.2	27.86 ±	0.95	18.0 ±	1.9
Polyunsaturated								
16:2n-4	1.15 ±	0.20	1.28 ±	0.48	2.46 ±	0.12	0.63 ±	0.15
16:3n-4	0.62 ±	0.12	0.90 ±	0.40	1.29 ±	0.12	0.67 ±	0.28
16:4n-3	0.58 ±	0.12	0.69 ±	0.12	0.46 ±	0.13	1.67 ±	0.34
16:4n-1	1.53 ±	0.31	3.2 ±	1.6	5.55 ±	0.54	1.07 ±	0.21
18:2n-6	1.20 ±	0.10	1.65 ±	0.27	0.86 ±	0.08	1.37 ±	0.08
18:2n-4	0.29 ±	0.03	0.24 ±	0.21	0.19 ±	0.05	0.28 ±	0.14
18:3n-6	0.12 ±	0.05	0.14 ±	0.10	0.36 ±	0.08	0.24 ±	0.08
18:3n-3	0.87 ±	0.19	1.16 ±	0.40	0.33 ±	0.06	0.96 ±	0.24
18:4n-3	3.52 ±	0.34	4.3 ±	1.2	1.74 ±	0.19	3.14 ±	0.50
18:4n-1	0.37 ±	0.09	0.12 ±	0.12	0.26 ±	0.11	0.27 ±	0.17
20:2n-6	0.24 ±	0.15	0.44 ±	0.27	0.31 ±	0.17	0.29 ±	0.05
20:3n-6	0.23 ±	0.16	0.16 ±	0.18	0.17 ±	0.06	0.06 ±	0.02
20:4n-6	0.36 ±	0.05	0.26 ±	0.08	0.30 ±	0.04	0.40 ±	0.06
20:4n-3	0.75 ±	0.02	0.80 ±	0.12	0.14 ±	0.23	0.88 ±	0.20
20:5n-3	16.52 ±	0.74	13.86 ±	0.79	22.8 ±	3.0	17.8 ±	1.5
21:5n-3	0.43 ±	0.07	0.26 ±	0.03	0.12 ±	0.12	0.33 ±	0.08
22:4n-6	0.01 ±	0.02	0.25 ±	0.24	0 ±	0	0.01 ±	0.02
22:5n-3	0.51 ±	0.16	0.55 ±	0.22	0.05 ±	0.09	0.78 ±	0.23
22.6n-3	14.2 ±	3.4	0.75 ±	0.20	3.06 ±	0.49	23.1 ±	2.0
Subtotal	43.5 ±	3.5	31.1 ±	1.6	40.4 ±	3.4	53.9 ±	1.6
Total	97.16 ±	0.33	83.7 ±	3.3	94.0 ±	1.4	95.10 ±	0.30

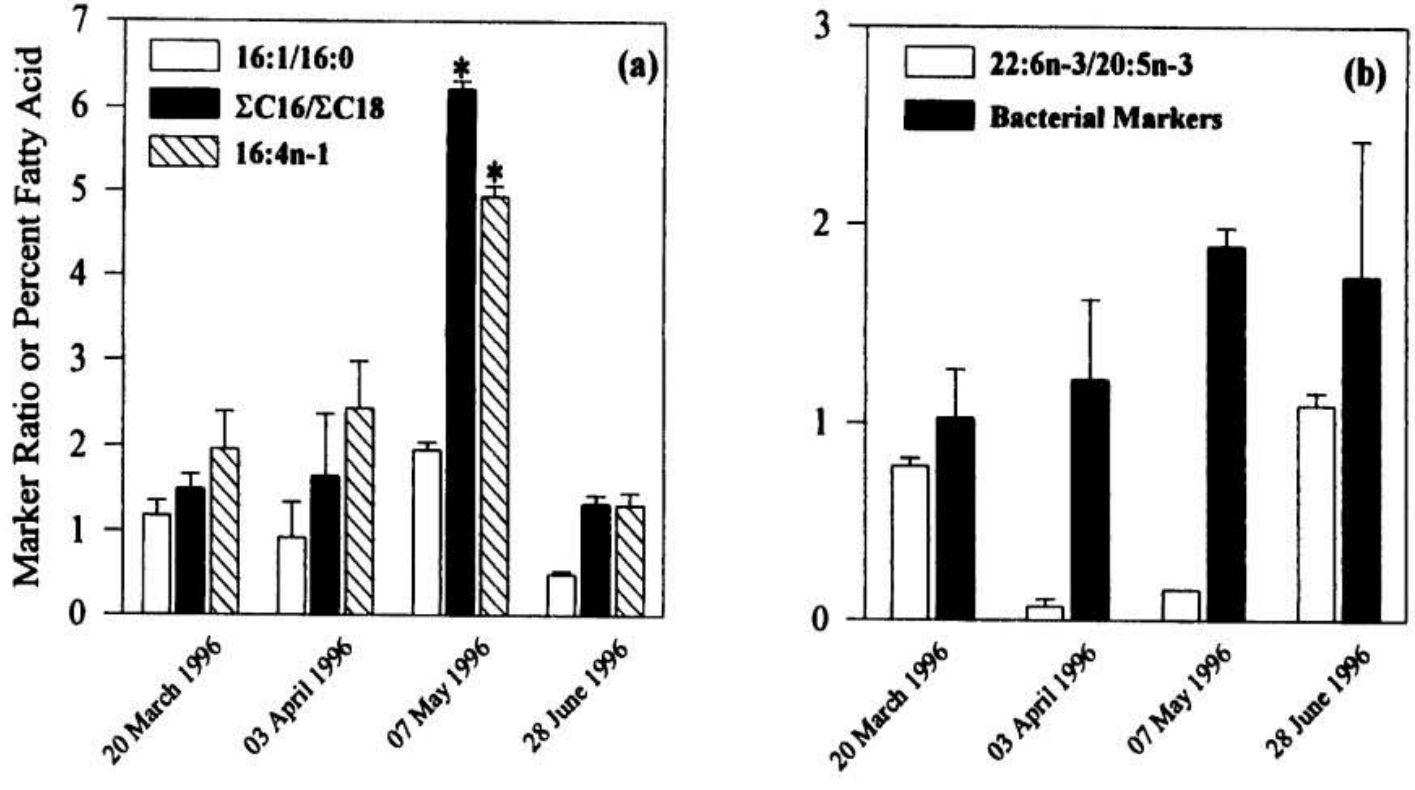


Figure 4.2 Values of various biomarkers in net-tows collected during the spring bloom of 1996 (mean ± standard deviation, n=3). (a) Diatom and (b) dinoflagellate (22:6n-3/20:5n-3) and bacterial (15:0 + 17:0 + branched chain fatty acids) indicators. An asterisk (*) indicates that data are significantly different ($P<0.05$) from all others in the graph.

at this site during the spring bloom of 1996 were typical of fresh marine phytoplankton (Volkman *et al*, 1989; Viso and Marty, 1993; Dunstan *et al*, 1994), containing elevated amounts of PUFA, particularly 18:4n-3, 20:5n-3 and 22:6n-3, and very low bacterial fatty acid proportions. The fatty acid biomarker ratios for marine sources previously described were applied to these samples.

The three separate diatom indicators (16:1/16:0, ΣC16/ΣC18 and 16:4n-1) agree well, showing elevated values during the spring bloom in May (Figure 4.2a), consistent with the predominance of diatoms, and lowest values in June. The ratio of 22:6n-3/20:5n-3 also reflected the relative abundance of diatoms versus dinoflagellates (Figure 4.2b). The value of this ratio was near unity in March and June when the 22:6n-3 producer, *Ceratium tripos*, was present and much less in April and May during the diatom bloom.

Finally, the lipid contribution of bacteria to the material collected in these net-tows was determined by examining levels of the bacterial indicator, defined as the sum of 15:0, 17:0 and branched chain fatty acids in percent of total fatty acids (Figure 4.2b). These values were all less than 2. A value of 1.5 has been found to correspond to a bacterial biovolume contribution of less than 0.1% in a culture of *Gymnodinium* cf. *nagasakiense* (Parrish *et al*, 1994). This suggests that bacteria were not an important source of fatty acids in these net-tows. However, in a recent paper, Harvey and Macko (1997) did not find a correlation between total fatty acids attributed to bacteria and bacterial carbon, and they suggest that bacterial fatty acids only be used as qualitative tools to estimate bacterial contributions. Wakeham (1995) also points out that fatty acids of common oceanic bacteria may not be

compositionally different from planktonic fatty acids so that bacterial contributions may not be easily discernable. On the other hand, Canuel and Martens (1993) proposes that bacterial biomass in coastal sediments can be calculated from bacterial markers such as those used here. In light of these conflicting theories, the remainder of the discussion concerning bacterial fatty acids will concentrate on bacterial levels relative only to other samples in this study. The value of these bacterial markers increased from March to May and seemed to reach a plateau in June. It is possible that bacteria increased in numbers and in importance as a source of fatty acid material simply because, as the bloom progressed, there was more organic matter available for oxidation and support of bacterial growth. Other studies in this area also report that bacteria are temperature sensitive and become more numerous as the water temperature increases in late spring and early summer (Pomeroy *et al*, 1991). However, these values were all quite low as compared to sediment trap and sediment samples (see below) and suggested that bacteria did not contribute a large portion of organic material to these net-tows.

4.3.3 Sediment Traps

Floristic examination of trap samples indicated large amounts of debris composed primarily of the skeletal remains of microalgae and a few zooplankton faecal pellets. Centric diatoms were the dominant living phytoplankton in all samples. The offshore trap that had been deployed during the winter and recovered on 11 April 1995 contained much larger amounts of *Coscinodiscus* sp. and probably captured the beginning of the spring bloom. Less than two weeks later, on 20 April 1995, *Coscinodiscus* sp. was not as numerous, and

Biddulphia aurita and other unidentified centric diatoms had become more important. Small amounts of pennate diatoms, including *Grammatophora* sp., *Fragilariopsis* sp. and *Nitzschia* sp., were present in most sediment traps throughout the year.

The organic material in these traps was analysed to determine fatty acid concentrations and fluxes. Few statistical differences were found in the fatty acid abundance and composition at the three trap depths (50, 75 and 100 m) so mean values for all traps at each site are presented in Table 4.5. The fatty acid compositions and fluxes during each sampling period were consistent with net-tow data and floristic examinations. The elevated fatty acid concentrations and fluxes found in the nearshore traps deployed from June to July and July to October were also consistent with the higher concentrations in the post-bloom period observed in the net-tow data. *In situ* chlorophyll measurements (Parrish, 1998) suggested that the bloom forms and sinks early in the offshore location and this is a probable explanation for the reduced concentration found in the offshore trap deployed in June. These traps were simply deployed too late to capture the sinking bloom in that year. The trap deployed from July to April had the lowest flux of all traps. Floristic analysis showed large numbers of the centric diatom, *Coscinodiscus* sp., in the trap and it is known that substantial material is produced in late March and April, before the spring bloom, so most of the material in the trap was likely derived from this March/April period. Finally, in April 1995, fatty acid fluxes in the nearshore and offshore traps began to increase in association with the beginning of the spring bloom.

Table 4.5 Concentrations and fluxes of total fatty acids and PUFA in settling particulate matter and in sediments. Units of concentrations and flux are μg g dry weight^{-1} and mg m^{-2} d^{-1}, respectively (mean ± standard deviation, n = 1-9).

Sampling Period and Location	Total Fatty Acids Conc		Flux		PUFA Conc		Flux	
Settling Particulate Matter								
20 June 94 - 20 July 94								
Offshore	5.20		4.6	± 3.0	0.84		0.80	± 0.39
20 June 94 - 19 July 94								
Nearshore	22	± 14	12.3	± 6.0	4.0	± 2.6	1.9	± 1.2
20 July 94 - 11 Apr 95								
Offshore	5.2	± 3.6	2.9	± 1.4	0.81	± 0.43	0.40	± 0.15
19 July 94 - 21 Oct 94								
Nearshore	21.6	± 8.4	10.7	± 4.7	3.2	± 1.3	1.56	± 0.71
11 Apr 95 - 20 Apr 95								
Offshore	3.1	± 2.3	8.6	± 6.1	0.54	± 0.37	1.54	± 0.92
11 Apr 95 - 20 Apr 95								
Nearshore	1.71	± 0.70	5.1	± 1.7	0.47	± 0.18	1.45	± 0.53
26 May 97 - 29 May 97								
Offshore	4.2	± 6.0	15	± 12	1.1	± 1.5	3.8	± 3.4
Sediment Trap Mean	8.4	± 10.3	8.8	± 7.4	1.7	± 1.9	1.9	± 1.9
Sediments								
Offshore	0.110	± 0.050	----------		0.008	± 0.005	----------	

The fatty acid composition of trap samples (Tables 4.6 and 4.7) were similar to the net-tows but the sediment trap samples contained significantly less PUFA ($P<0.001$) and more saturated and monounsaturated fatty acids. Degradation by bacteria is a possible cause. All sediment trap samples contained diatoms as the dominant phytoplankton. As a result, little variation was seen in the values of diatom indicators (Figure 4.3). The marker ratios also suggested that most of the debris in these traps was of diatom origin. The amount of 16:4n-1 found in the samples was larger in the late April traps which contained *Biddulphia aurita* and the unidentified centric diatoms, in addition to *Coscinodiscus* sp. It is possible that *Biddulphia aurita* and these unidentified centrics contained a larger proportion of 16:4n-1 than did *Coscinodiscus*. In all sediment trap samples, the three indicators had values similar to those of the net-tows taken before and after the spring bloom, probably reflecting the fact that none of these sediment traps deployed in 1994 and 1995 captured the peak of the spring bloom.

Similarly, little variation was observed in the dinoflagellate marker, 22:6n-3/20:5n-3, (Figure 4.4) which remained near 0.5 and suggested that dinoflagellates were not an important source. This is in agreement with the floristic examination that did not reveal dinoflagellates in any of the sediment trap samples examined. Terrestrial indicators also displayed few significant differences among the traps. Values of this marker were quite low and suggested that terrestrial lipid inputs to this marine system were small. Only the sample collected in the fall had a value approaching 2.5, indicating a substantial input of terrestrial lipids. Terrestrial material may be a relatively more important source in the fall and winter simply because there are fewer phytoplankton-derived fatty acids present to dilute the signal.

Table 4.6 Fatty acid composition (% total fatty acids) of nearshore sediment traps (mean ± standard deviation, n=8-9). Concentrations are given in Table 4.5.

Fatty Acid	20 June 1994-19 July 1995	20 July 1994-21 October 1994	11 April 1995-20 April 1995
Branched			
i-15:0	0.87 ± 0.21	1.47 ± 0.31	0.67 ± 0.11
ai-15:0	0.59 ± 0.38	0.99 ± 0.31	0.67 ± 0.56
i-16:0	0.32 ± 0.24	0.58 ± 0.02	0.83 ± 0.47
Subtotal	1.78 ± 0.82	3.04 ± 0.59	2.17 ± 0.99
Saturated			
14:0	9.4 ± 1.8	15.8 ± 2.5	7.03 ± 0.78
15:0	0.96 ± 0.09	1.86 ± 0.17	0.84 ± 0.23
16:0	17.4 ± 3.6	15.2 ± 1.1	13.6 ± 1.8
17:0	0.53 ± 0.16	0.48 ± 0.17	0.77 ± 0.26
18:0	1.49 ± 0.17	1.32 ± 0.35	3.1 ± 1.0
20:0	0.01 ± 0.02	0.09 ± 0.09	0.24 ± 0.11
22:0	0 ± 0	0.02 ± 0.03	0.17 ± 0.11
23:0	0.10 ± 0.17	0 ± 0	0 ± 0
24:0	0 ± 0	0.04 ± 0.04	0.19 ± 0.21
Subtotal	29.9 ± 5.1	34.8 ± 1.8	26.0 ± 2.5
Monounsaturated			
16:1n-7	18.5 ± 4.8	9.5 ± 1.2	12.40 ± 0.16
16:1n-5	0.40 ± 0.33	0.98 ± 0.51	0.48 ± 0.22
18:1n-9	12.76 ± 0.92	10.0 ± 1.6	10.5 ± 2.1
18:1n-7	1.0 ± 1.1	1.0 ± 1.5	0.91 ± 0.79
18:1n-5	1.12 ± 0.58	0.74 ± 0.14	0.59 ± 0.28
20:1n-11	4.7 ± 1.5	2.3 ± 1.8	0.08 ± 0.15
20:1n-9	1.4 ± 1.3	2.73 ± 0.59	2.0 ± 1.0
20:1n-7	0.55 ± 0.32	0.39 ± 0.13	0.66 ± 0.31
22:1n-11	3.9 ± 1.5	4.9 ± 1.9	1.18 ± 0.64
22:1n-9	0.20 ± 0.20	0.08 ± 0.07	0.26 ± 0.20
24:1	0.86 ± 0.48	0.70 ± 0.11	0.97 ± 0.73
Subtotal	45.4 ± 4.9	33.4 ± 5.8	30.0 ± 1.7
Polyunsaturated			
16:2n-4	0.73 ± 0.13	0.94 ± 0.39	1.25 ± 0.77
16:3n-4	0.79 ± 0.18	1.10 ± 0.25	1.38 ± 0.58
16:4n-3	0.26 ± 0.03	0.58 ± 0.15	0.67 ± 0.18
16:4n-1	0.72 ± 0.25	0.38 ± 0.15	2.52 ± 0.31
18:2n-6	0.95 ± 0.33	1.26 ± 0.10	1.23 ± 0.53
18:2n-4	0.33 ± 0.03	0.71 ± 0.28	0.24 ± 0.05
18:3n-6	0.08 ± 0.06	0.20 ± 0.07	0.38 ± 0.20
18:3n-3	0.54 ± 0.19	1.00 ± 0.14	0.61 ± 0.12
18:4n-3	1.99 ± 0.28	2.56 ± 0.32	2.01 ± 0.08
18:4n-1	0.14 ± 0.09	0.03 ± 0.06	0.47 ± 0.81
20:2n-6	0.23 ± 0.08	0.28 ± 0.07	0.38 ± 0.15
20:3n-6	0.06 ± 0.07	0 ± 0	0.37 ± 0.64
20:4n-6	0.01 ± 0.02	0.03 ± 0.04	0.09 ± 0.04
20:4n-3	0.42 ± 0.09	0.47 ± 0.01	0.42 ± 0.16
20:5n-3	6.02 ± 0.28	7.0 ± 5.5	11.0 ± 1.1
21:5n-3	0.37 ± 0.14	0.10 ± 0.06	0.39 ± 0.42
22:4n-6	0 ± 0	0.01 ± 0.01	0.02 ± 0.04
22:5n-3	0.51 ± 0.09	0.31 ± 0.09	0.86 ± 0.71
22:6n-3	3.50 ± 0.68	2.22 ± 0.39	7.0 ± 1.2
Subtotal	17.6 ± 1.2	19.2 ± 5.6	31.4 ± 3.1
Total	94.76 ± 0.49	90.5 ± 2.5	89.6 ± 1.2

Table 4.7 Fatty acid composition (% total fatty acids) of offshore sediment traps (mean ± standard deviation, n=9). Concentrations are given in Table 4.5.

Fatty Acid	20 June 1994-20 July 1994	20 July 1994-11 April 1995	11 April 1995-20 April 1995
Branched			
i-15:0	1.02 ± 0.71	2.33 ± 0.44	0.77 ± 0.08
ai-15:0	1.13 ± 0.47	2.20 ± 0.60	0.78 ± 0.27
i-16:0	0.51 ± 0.37	0.49 ± 0.08	0.76 ± 0.48
Subtotal	2.66 ± 0.51	5.0 ± 1.1	2.31 ± 0.80
Saturated			
14:0	8.7 ± 1.2	12.7 ± 4.6	7.15 ± 0.39
15:0	1.04 ± 0.23	1.59 ± 0.33	1.05 ± 0.19
16:0	15.0 ± 3.8	16.7 ± 2.6	17.8 ± 1.2
17:0	0.29 ± 0.13	0.22 ± 0.21	0.54 ± 0.13
18:0	2.32 ± 0.10	1.46 ± 0.23	4.2 ± 1.4
20:0	0.04 ± 0.07	0.16 ± 0.05	0.10 ± 0.02
22:0	0.01 ± 0.02	0.14 ± 0.10	0.21 ± 0.07
23:0	0 ± 0	0 ± 0	0.14 ± 0.02
24:0	0.17 ± 0.15	0.09 ± 0.10	0.21 ± 0.15
Subtotal	27.5 ± 4.7	33.0 ± 6.7	31.4 ± 2.0
Monounsaturated			
16:1n-7	18.3 ± 2.5	14.9 ± 1.7	12.4 ± 3.6
16:1n-5	0.64 ± 0.97	1.84 ± 0.09	0.53 ± 0.20
18:1n-9	8.4 ± 2.5	4.70 ± 0.88	10.3 ± 3.2
18:1n-7	1.6 ± 2.1	4.6 ± 1.1	1.7 ± 1.2
18:1n-5	0.80 ± 0.50	0.92 ± 0.29	0.55 ± 0.08
20:1n-11	2.6 ± 2.6	0.15 ± 0.26	0.22 ± 0.20
20:1n-9	2.2 ± 3.8	2.9 ± 1.1	3.03 ± 0.75
20:1n-7	0.35 ± 0.12	0.38 ± 0.06	1.15 ± 0.41
22:1n-11	3.4 ± 2.1	2.4 ± 1.4	1.89 ± 0.54
22:1n-9	0.17 ± 0.19	0.29 ± 0.26	0.34 ± 0.31
24:1	0.92 ± 0.46	0.60 ± 0.32	0.84 ± 0.35
Subtotal	39.4 ± 4.2	33.7 ± 1.1	32.9 ± 3.7
Polyunsaturated			
16:2n-4	0.87 ± 0.12	1.18 ± 0.14	1.40 ± 0.56
16:3n-4	0.89 ± 0.21	0.73 ± 0.60	0.90 ± 0.21
16:4n-3	0.23 ± 0.13	0.48 ± 0.17	0.39 ± 0.10
16:4n-1	0.99 ± 0.41	0.65 ± 0.34	1.74 ± 0.39
18:2n-6	0.88 ± 0.08	1.18 ± 0.13	0.88 ± 0.29
18:2n-4	0.48 ± 0.45	0.68 ± 0.25	0.21 ± 0.13
18:3n-6	0.27 ± 0.17	0.27 ± 0.09	0.02 ± 0.03
18:3n-3	0.46 ± 0.16	0.61 ± 0.03	0.67 ± 0.32
18:4n-3	1.55 ± 0.20	1.27 ± 0.02	1.49 ± 0.54
18:4n-1	0 ± 0	0 ± 0	0.03 ± 0.06
20:2n-6	0.20 ± 0.14	0.25 ± 0.04	0.47 ± 0.10
20:3n-6	0.38 ± 0.34	0.02 ± 0.02	0.09 ± 0.08
20:4n-6	0.15 ± 0.13	0.65 ± 0.34	0.05 ± 0.08
20:4n-3	0.35 ± 0.16	0.23 ± 0.04	0.42 ± 0.16
20:5n-3	9.3 ± 3.0	6.9 ± 2.5	8.4 ± 2.0
21:5n-3	0.09 ± 0.16	0.09 ± 0.06	0.29 ± 0.05
22:4n-6	0 ± 0	0 ± 0	0.18 ± 0.14
22:5n-3	0.60 ± 0.13	0.43 ± 0.27	0.24 ± 0.07
22:6n-3	4.8 ± 1.5	3.7 ± 1.5	6.4 ± 1.5
Subtotal	22.4 ± 4.7	19.3 ± 5.6	24.3 ± 3.8
Total	92.0 ± 3.4	90.96 ± 0.99	90.9 ± 1.3

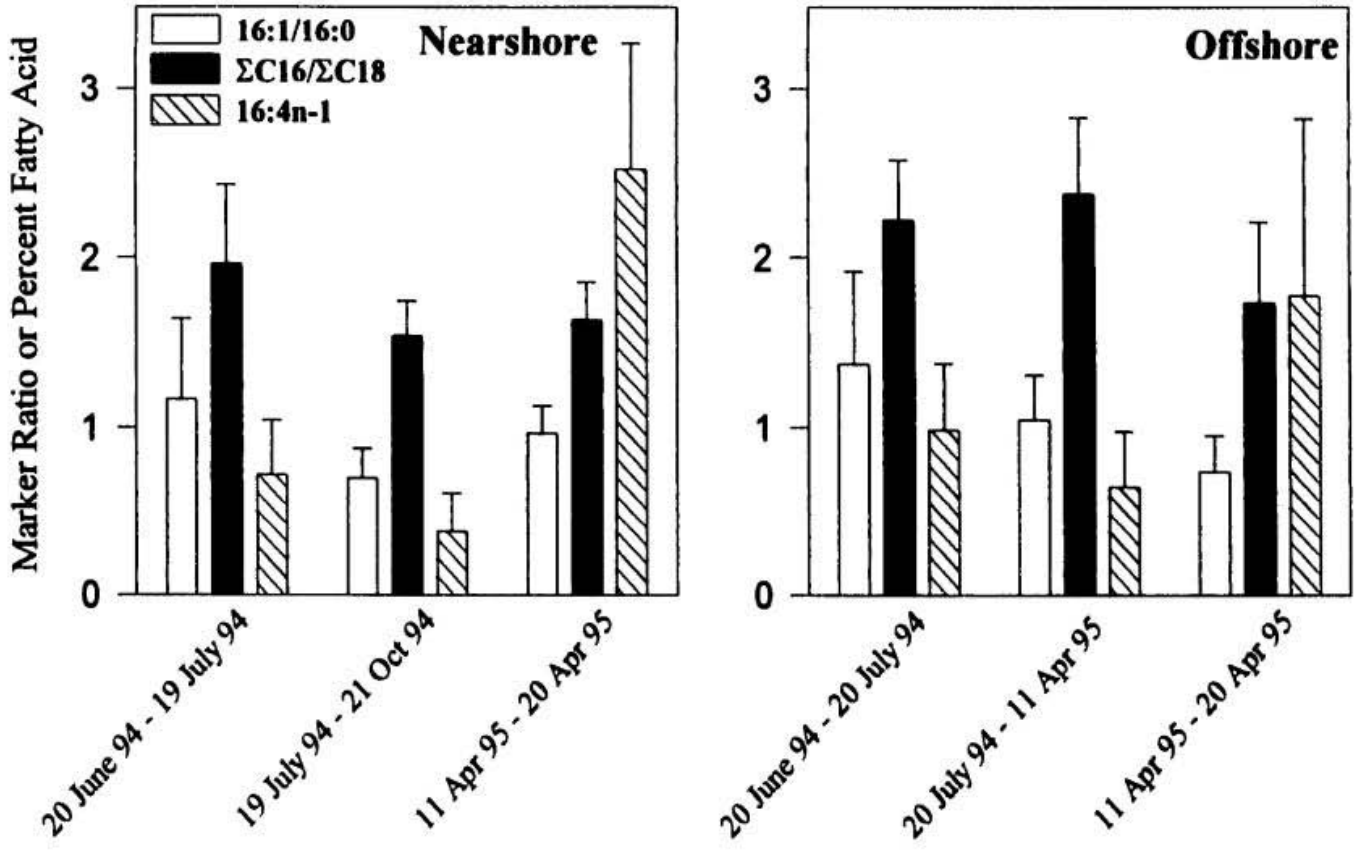


Figure 4.3 Values of diatom indicators in nearshore and offshore sediment traps (mean ± standard deviation, n=8-9).

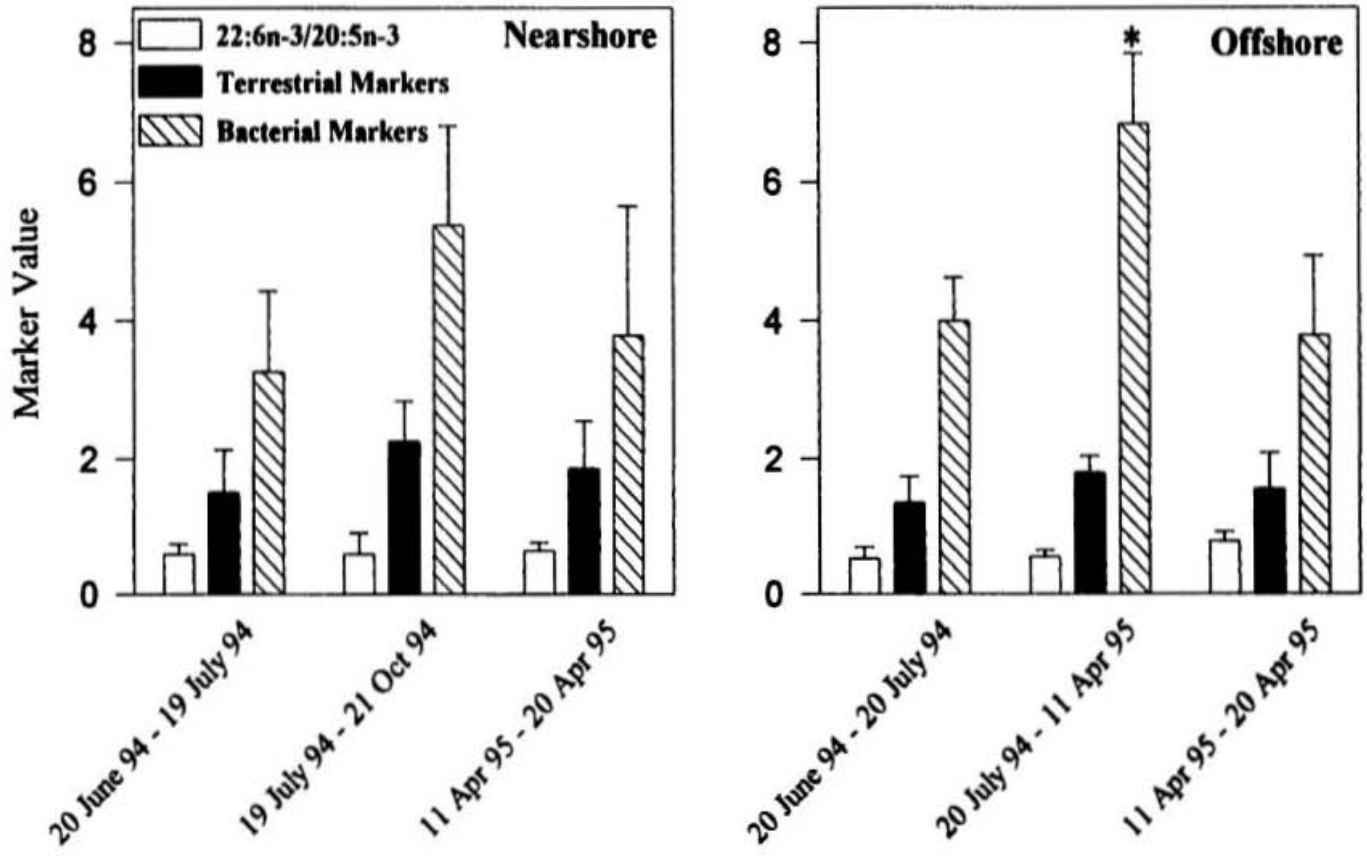


Figure 4.4 Values of dinoflagellate (22:6n-3/20:5n-3), terrestrial (18:2n-6+18:3n-3) and bacterial (15:0 + 17:0 + branched chain fatty acids) markers in nearshore and offshore sediment traps (mean ± standard deviation, n=8-9). An asterisk (*) indicates that data are significantly different ($P<0.05$) from all others in the graph.

The application of bacterial markers to the sediment traps is also shown in Figure 4.4. As the period of trap deployment increases, the period that collecting particles in the sediment trap are exposed to bacteria also increases. It is also possible that bacteria can be introduced to sediment traps through association with settling particulate matter. For both of these reasons, more bacterial markers would be expected in the fall and winter traps which had a longer deployment period than the traps deployed in spring and summer. These bacterial compounds may also be associated with faecal pellets which would be more common after the bloom (Rivkin *et al*, 1996). It is clear from Figure 4.4 that the bacterial markers in the fall and winter traps were more abundant than those in the other traps. In most cases, these values were all twice as high as those found in the net-tows and the concern is that these markers may be of sufficient value to indicate substantial bacterial degradation of lipid material. Wakeham and Beier (1991) used branched chain fatty acids, in addition to other fatty acids, to indicate bacterial activity and suggested that such activity was low in traps where branched chain fatty acids comprised approximately 6% of total. The largest marker value obtained in the current study was a value of 7 and those traps will be considered as having bacteria as only a minor source of fatty acids. In addition, Pomeroy *et al* (1991) found that both the sub-zero temperatures of these waters and the lack of necessary bacterial substrates produced through zooplankton metabolism combine to limit bacterial biomass to less than 5% of that normally encountered in coastal waters.

A separate array of sediment traps was deployed at the offshore site in May of 1997 to determine the effects of poisoning and dissolution on the fatty acid composition. Fatty acid

fluxes in these traps were the largest encountered in this study with a mean of 14.7 ± 11.5 mg m^{-2} d^{-1} (Table 4.5). It is likely that this trap was deployed at a more opportune time and collected organic matter derived from the peak of bloom growth rather than pre- and post-bloom material as captured by the other traps.

In the May deployment, one sampling cylinder of every four was poisoned with mercuric chloride at a concentration of 5 g L^{-1} which is in excess of that recommended by Lee *et al* (1992). As expected, these poisoned cylinders did capture many more zooplankton swimmers than non-poisoned traps and these swimmers had to be removed from the samples with forceps. Fatty acid analyses of the material in these traps and a comparison of the composition of poisoned and non-poisoned cylinders were carried out. No significant differences were found in the fatty acid composition, including total PUFA and bacterial markers. The mean bacterial indicator value in these traps was also quite low with a value of approximately 2, implying that bacterial biomass was low and supporting the conclusions of Pomeroy *et al* (1991) that bacterial activity is limited in the spring in this area. This suggests that, in this environment, bacterial degradation in sediment traps does not have sufficient impact on lipid data to warrant the use of poisons in short-term deployments.

In the bottom litre of both poisoned and non-poisoned cylinders, mean fatty acid concentrations in the particulate and dissolved fractions were 257 ± 216 μg L^{-1} and 218 ± 196 μg L^{-1}, respectively, supporting the results of Körtzinger *et al* (1994) who found dissolved fatty acids contributing up to 75% of the total flux. The dissolved concentrations in the cylinders can be compared with concentrations in nearby Conception Bay, NF (Liu *et al*,

1998) of 12 to 40 $\mu g\ L^{-1}$. Thus, at most, only about 20% of the dissolved fatty acids in this study can be attributed to natural background levels. These traps were also spiked with 4 L of 40 ppt NaCl which formed a barrier to prevent mixing of trap material with surrounding seawater containing dissolved lipids. On recovery, a salinity check did confirm that little dilution, and hence mixing, of this 40 ppt solution occurred. This suggests that most of the dissolved fatty acids do, in fact, stem from particle leaching. However, particle aggregates, such as marine snow, may also be delivering dissolved lipids to the traps in their interstitial waters. It should also be noted that the values given here do not consider diffusive losses. Hence, the levels of dissolved fatty acids in this study may actually be a conservative estimate. Few data concerning solubilities of lipids in salt water exist but examination of other data reporting large dissolved lipid concentrations (Bhosle *et al*, 1983) suggests that the dissolved concentrations measured in this study are approaching the solubility maximum. It seems unlikely that hydrophobic lipids would be capable of dissolving to a much greater extent than this in sea water. If the solubility maximum is being approached, application of the principles of equilibrium chemistry imply that increases in the particulate lipid concentration should result in only slight increases in the dissolved lipid concentration. As a result, losses of lipid material to dissolution would become proportionately less of a concern as the period of trap deployment and concentration of particulate lipid increases. However, in that case, bacterial activity would be expected to increase, resulting in the metabolism of these dissolved lipids and reduction of their concentrations. This would disturb any chemical equilibria that may have been established and could result in the dissolution of even more lipids.

The relative fatty acid composition of the dissolved portion was also determined and key aspects of the fatty acid distribution in the dissolved fraction were found to be significantly different from those in the particulate fraction (Table 4.8). Most noticeably, the dissolved fraction contained fewer PUFA and the average fatty acid was shorter in chain length and more saturated. Körtzinger *et al* (1994) also reported similar results and suggested that the smaller amounts of long chain fatty acids in the dissolved portion might simply reflect the decreasing water solubility of lipids as chain length increases. These PUFA are often associated with membranes and it may be that PUFA are more tightly bound in cellular structures and are not as readily available for dissolution as the more saturated acids. If bacteria are consuming this dissolved material, lower PUFA levels may simply be a result of preferential uptake of those fatty acids.

4.3.4 Sediments

Surficial sediments at the offshore sampling site (St-7) were also examined for fatty acid content. Eight samples from a sediment core were examined but no significant differences were found among the fatty acid concentrations in each horizon. Because of this, the mean of all core data is reported as sediment results with no distinction as to core depth. The mean fatty acid concentration was 0.110 ± 0.050 mg g dry weight^{-1} (Table 4.5) and the fatty acid composition and concentration were comparable to that found in other areas of high fluxes of organic material to the sediments (Harvey, 1994; Colombo *et al*, 1997). The samples were

Table 4.8 Parameters of the particulate and dissolved fractions of sediment trap material collected in May of 1997 (mean ± standard deviation, n=6-11). PUFA and saturated acid data are percent total fatty acids. Chain lengths and double bonds are mean number of carbon atoms and double bonds, respectively, in each fatty acid, calculated using mole percent of total fatty acids.

	Particulate Fraction	Dissolved Fraction	P value
PUFA	29.0 ± 3.2	20.4 ± 4.9	<0.001
Saturated Acids	28.4 ± 3.5	34.3 ± 6.1	<0.024
Chain Length	17.34 ± 0.14	16.37 ± 0.37	<0.001
Double Bonds	1.67 ± 0.13	1.20 ± 0.21	<0.001

dominated by saturated and monounsaturated acids (Table 4.9) and contained the largest amount of bacterial fatty acids encountered in any sample (9.8 ± 1.8%). PUFA comprised only 7.8% and the terrestrial indicators, 18:2n-6 and 18:3n-3, made up the bulk of this. Only 0.5% by weight of the PUFA concentration found in the traps was preserved in the sediments. A portion of such fatty acid losses are commonly attributed to alteration of fatty acids at the sediment-water interface or in surficial sediments by diagenetic processes (Haddad *et* al, 1992; Canuel and Martens, 1996). The high levels of bacterial markers in the sediments indicate the importance of bacteria as a source of fatty acid material and suggest that bacterial degradation of PUFA may be an important process.

4.3.5 Biogeochemical Cycling

To date, the fatty acid fluxes measured in the Laurentian Trough at a seaward station (Colombo *et al,* 1996a) of approximately 5.5 ± 2.9 mg m^{-2} d^{-1} are the highest reported in and around the north Atlantic. The Laurentian Trough is at the same latitude as Trinity Bay, NF and is a comparable environment, although the Laurentian Trough does have a large input of terrestrial material (Colombo *et al*, 1996b). However, even with this added terrestrial input, fatty acid fluxes measured during May and July in the Laurentian Trough are still less than the mean fluxes recorded in Trinity Bay in 1994, 1995 and 1997 of 8.8 ± 7.4 mg m^{-2} d^{-1}. A comparison of the May data sets from this study and that of Colombo *et al* (1996a), both of which sampled the spring diatom bloom, shows that the fatty acid fluxes in Trinity Bay in May 1997 (15 ± 12 mg m^{-2} d^{-1}) are more than twice as large as the fatty acid fluxes recorded by

Table 4.9 Fatty acid composition (% total fatty acids) of offshore sediments (mean ± standard deviation, n=8). Concentrations are given in Table 4.5.

Fatty Acid		
Branched		
i-15:0	1.27 ±	0.20
ai-15:0	4.90 ±	0.97
i-16:0	0.43 ±	0.29
Subtotal	6.6 ±	1.3
Saturated		
14:0	6.26 ±	0.68
15:0	3.18 ±	0.80
16:0	20.7 ±	1.8
18:0	6.6 ±	1.5
20:0	0.01 ±	0.04
22:0	0.16 ±	0.20
Subtotal	43.2 ±	2.1
Monounsaturated		
16:1n-7	14.8 ±	2.6
18:1n-9	4.8 ±	2.2
18:1n-7	4.2 ±	2.1
18:1n-5	0.04 ±	0.12
20:1n-9	3.1 ±	1.2
20:1n-7	0.10 ±	0.19
22:1n-11	3.38 ±	0.77
24:1	0.94 ±	0.87
Subtotal	31.4 ±	3.7
Polyunsaturated		
18:2n-6	2.90 ±	0.99
18:2n-4	0.11 ±	0.15
18:3n-3	2.81 ±	0.69
18:4n-3	0.30 ±	0.20
20:2n-6	0.09 ±	0.22
20:3n-6	0.11 ±	0.16
20:4n-6	0.59 ±	0.59
20:4n-3	0.09 ±	0.14
20:5n-3	0.77 ±	0.19
Subtotal	7.77 ±	0.63
Total	88.9 ±	4.1
Bacterial Acids	9.8 ±	1.8
Terrestrial Acids	5.71 ±	0.76

Colombo *et al* (1996a) in May (6.6 ± 4.0 mg m^{-2} d^{-1}). In addition, fluxes of PUFA in May 1997 in Trinity Bay of 3.8 ± 3.4 mg m^{-2} d^{-1} are significantly higher (P=0.0048) than the corresponding fluxes in the Laurentian Trough of 0.40 ± 0.43 mg m^{-2} d^{-1}. This information implies that the water column in Trinity Bay is more productive, producing larger fluxes of fatty acids and in particular, PUFA, than the Laurentian Trough.

Normally, extensive losses of PUFA would be expected during sedimentation in the water column by bacterial degradation and uptake by higher organisms. In an adjacent bay, Conception Bay (Figure 4.1), sedimentation rates for particles were approximately 20-23 m d^{-1} (Redden, 1994) which corresponds to a residence time in the water column of only 15 days at typical sampling sites (approximate depths of 300 m). Harvey *et al* (1995) studied the kinetics of phytoplankton decay in oxic water at 19 °C and suggested a rate constant of 8.3 y^{-1} in the decay relationship $G_t = G_o e^{-kt}$ where G_o is the original lipid concentration, G_t is the lipid concentration at any time t, and k is the rate constant. For a settling time of 15 days, this results in roughly a 30% loss of lipid material. However, in this study, water temperatures are generally sub-zero which, according to chemical kinetic theory, would produce a four fold decrease in the rate constant. This produces losses in the water column that are closer to 10% and implies that most of the fatty acid material produced in surface waters could reach the sediment-water interface intact and undegraded. In contrast, Colombo *et al* (1997) report lipid losses of up to 93% in the water column between 150 m and the interfacial sediments in the Laurentian Trough.

Assuming that the above argument is correct and only a 10% loss of fatty acid material occurs in the water column, then less then 1% of the fatty acids that reach the sediment-water interface are preserved in the sediments and diagenetic processes are obviously responsible for these huge losses. A variety of rate constants describing the degradation of fatty acids in oxic sediments exist (Haddad *et al*, 1992; Henrichs, 1992; Canuel and Martens, 1996) and most describe a rapid reduction in fatty acid concentrations within a period of two years or less after deposition. PUFA concentrations were even more depleted than that of total fatty acids, possibly because of the high lability of unsaturated acids. Benthic macrofaunal organisms also consume large amounts of organic material and have an absolute requirement of PUFA for growth and survival. In this environment, they are known to contain high levels of PUFA (Parrish *et al*, 1996b) and it is likely that they have a significant role in the reworking of fatty acid material at the sediment-water interface. These organisms, as well as bacteria, are capable of contributing fatty acids to the sediments, in addition to consuming that material. It should be noted that approximately 73% of the PUFA preserved in the sediments was composed of the terrestrial indicators proposed here, 18:2n-6 and 18:3n-3. Numerous studies (Haddad *et al*, 1992; Harvey, 1994; Santos *et* al, 1994; Canuel and Marten, 1996; Colombo *et* al, 1997) have reported that fatty acids derived from terrestrial plants, generally long-chain saturates, were preserved to a greater degree than marine-derived acids. This suggests that fatty acids of marine origin, rather than terrestrial, are being selectively removed from the water column. Marine acids are generally more unsaturated than terrestrial acids and it is likely that they are preferentially incorporated by marine organisms. It is also

possible that these terrestrial acids have simply been "packaged" in particles in such a way as to make them inaccessible to bacteria (Haddad *et al*, 1992). In any case, the high flux of marine PUFA and minor preservation in sediments suggests that the benthic community has an ample supply of lipids of high nutritional value.

4.4 Conclusions

The fatty acid biogeochemistry of a fjord-like bay in eastern Newfoundland, Canada, was investigated to determine sources and fates of organic material. Samples of plankton, settling particulate matter and sediments were examined and fatty acids were used to identify their sources. Centric diatoms, as well as the dinoflagellate *Ceratium tripos*, were the dominant phytoplankton, producing, through primary production, large fluxes of settling particulate matter. Mean fatty acid fluxes of 8.8 ± 7.4 mg $m^{-2} d^{-1}$ were produced in 1994, 1995 and 1997. Fatty acid distributions suggested that the acids were almost totally marine in origin with very few terrestrial plant indicators present and that bacterial biomass, as indicated by odd carbon numbered and branched chain fatty acids, seemed to be low. Short-term sediment trap studies with mercuric chloride poisoning did not reveal a significant difference in fatty acid composition in traps with and without poison; however, dissolution of fatty acids from particles can introduce large errors in flux and concentration measurements. PUFA concentrations in sediments were less than 0.5% of the concentration found in settling particulate matter. Fatty acids of bacterial and terrestrial origin were present at higher concentrations in the sediments and it appears that the fatty acids of marine origin are

effectively recycled by diagenetic processes near the sediment-water interface. This implies that, in terms of fatty acid nutrients, the lower food web in this area is very efficient as there is a minimal loss of the high flux of fatty acids through burial in sediments.

Chapter 5 - LIPID CLASS AND FATTY ACID COMPOSITION OF *PSEUDO-NITZSCHIA MULTISERIES* AND *PSEUDO-NITZSCHIA PUNGENS* AND EFFECTS OF LIPOLYTIC ENZYME DEACTIVATION

5.1 Introduction

Lipid analyses of toxic algae can reveal signature compounds that may be useful in screening water or seafood samples. These signature compounds or biomarkers are often molecules with unusual structures that can be used to establish the presence of the alga or of organisms that have consumed the alga. The pennate diatom, *Pseudo-nitzschia multiseries*, is a domoic acid producer which was responsible for an outbreak of amnesic shellfish poisoning in Canada (Addison and Stewart, 1989). Its morphology is very similar to that of its relative, *Pseudo-nitzschia pungens*, which was thought to be non-toxic (Bates *et al*, 1993); however, recent data have shown this organism to also be a domoic acid producer (Rhodes *et al*, 1996). The two organisms have overlapping measurements of length, width and number of costae (rib-like structures) per unit length (Hasle *et al*, 1996). The only differentiating feature is the number of pores between these costae, and electron microscopy is necessary to view this difference. Chemotaxonomic differences, particularly regarding lipids, may be useful in differentiating toxic *Pseudo-nitzschia* species from other diatoms. Domoic acid itself could be used as an indicator of the toxic algae, but procedures for domoic acid determination (Quilliam *et al*, 1995) are rather time consuming and expensive. The aquaculture industry often determines fatty acid composition in their products for nutritional information so a fatty

acid biomarker that could be detected in the same manner would be useful. For example, the fatty acid, 16:4n-1, has been suggested as a signature compound of *P. multiseries* (Parrish *et al*, 1991), and it is known that proportions of this acid fluctuate with scale of culture (Whyte *et al*, 1995). This study was undertaken to evaluate the usefulness of 16:4n-1 and other fatty acids as biomarkers for *P. multiseries*.

A second objective was to determine the extent of degradation of acyl lipids by lipolytic enzymes. The effects of *in vitro* hydrolysis on the lipid composition of the diatom, *Skeletonema costatum*, were recently examined (Berge *et al*, 1995) and were found to be responsible for reduced amounts of glycolipids and excesses of FFA. However, the addition of boiling water to the sample after centrifugation effectively deactivated lipolytic enzymes. A similar approach, using boiling water treatment to denature those enzymes, was applied here to *P. multiseries* and *P. pungens* cultures to observe changes in lipid composition due to lipase activity. Diatoms also contain lipoxygenases (Gerwick, 1994) which are responsible for PUFA degradation and act only on FFA (Erikson, 1987). Boiling water treatment was also expected to affect these enzymes and result in altered fatty acid compositions.

5.2 Experimental

Cultures of *Pseudo-nitzschia multiseries* (CCMP 1660) and *P. pungens* (CCMP 1572) were obtained from the Bigelow Lab, ME, USA. They were grown in f/2 medium (Guillard and Ryther, 1962) enriched with Se (0.01 μmol L^{-1}), and during a 37 day growth period, they were transferred to new medium in a larger flask and enriched with nutrients 3

times. Illumination was provided on a 14:10 light:dark cycle at 15 °C. When the cultures of *P. multiseries* and *P. pungens* reached early stationary phase with growth rates of 0.01 divisions d^{-1} and 0.04 divisions d^{-1}, respectively, approximately 3/4 of the cultures were harvested and divided into 6 or 8 equal fractions. At this time, the cultures had concentrations of 1.95 X 10^6 cells mL^{-1} and 1.08 X 10^6 cells mL^{-1}, respectively. Each fraction was then filtered on precombusted GF/C glass fibre filters. Immediately after filtration, 10 ml of boiling distilled H_2O was poured over half of the filtered cells, and an instant colour change from greenish-brown to bright yellow was observed. Lipids were extracted as described in Section 3.2.3.1. Lipid class and fatty acid compositions were also determined as in Sections 3.2.4.1 and 3.2.6.1, respectively.

The remaining portion of these cultures were then transferred to new media with half the silica concentration and were grown for a further 26 days without transfer or nutrient replenishment. After harvesting, these cultures were treated in the same manner as the first fractions. At this time, it was not possible to count the cells as many of the *P. multiseries* cells were clumped, while many of the *P. pungens* cells were in chains. There was also a significant amount of detritus in both cultures. These microscopic observations plus evidence of spore formation indicate that the cultures were senescent. All stationary phase and senescent cultures were silica limited with silicate levels of only 20% of those of the original medium, while nitrate and phosphate concentrations were greater than 80% of the original.

Cultures of diatoms were also obtained from the National Research Council of Canada, Halifax, NS, for use in a secondary study. *P. multiseries* (KP 105) was grown in L1

medium (Guillard and Hargraves, 1993) enriched with Si. As with the first strain, light was provided on a 14:10 light:dark cycle at a temperature of 16 °C. Growth rates and cell counts were not available for these cultures but portions were harvested in both stationary and senescent phases. Those samples were divided into two equal fractions, one of which was treated with boiling water after filtration. Nutrient analyses indicated that these cultures were also silicate limited. Lipid class and fatty acid compositions were determined in these cultures as described above.

5.3 Results and Discussion

5.3.1 Lipolytic Enzyme Deactivation

Tables 5.1 and 5.2 display the lipid class composition of all cultures with and without boiling water treatment prior to extraction. FFA proportions in all treated cultures were less than half those found in the corresponding untreated samples. Absolute amounts of FFA per cell were also significantly lower ($P<0.05$) in the treated cultures. This indicates that boiling water treatment did deactivate lipolytic enzymes. In both cultures harvested in early stationary phase (Table 5.1), the untreated samples contained higher levels of FFA and AMPL, which include the breakdown product MAG, and lower levels of PL than treated samples. With boiling water treatment, however, proportions of FFA and AMPL were substantially decreased with corresponding increases in PL. Obviously, PL, rather than TAG, was the source of the FFA and MAG. This is in agreement with the results from *Skeletonema costatum* (Berge *et al*, 1995) and indicates that the high levels of FFA reported in other

Table 5.1 Lipid class composition and concentration of early stationary phase cultures of *P. pungens* and *P. multiseries*. Lipid class compositions are reported as percent of total lipids detected. Lipid concentrations are in pg cell^{-1} (mean ± standard deviation, n=3).

	P. multiseries		*P. pungens*	
	Treated	Untreated	Treated	Untreated
Total Lipids (conc)	2.7 ± 0.4	3.6 ± 0.8	6.5 ± 0.7	6.3 ± 0.5
Lipid Class				
Hydrocarbons	15 ± 12	3.9 ± 2.9	9.1 ± 2.3	1.1 ± 1.3
Steryl/Wax Esters	0.2 ± 0.2	0.8 ± 0.4	1.1 ± 0.1	2.3 ± 2.4
Methyl Esters	2.2 ± 2.0	—	—	—
Ethyl Ketones	4.1 ± 0.6	3.8 ± 0.7	2.9 ± 0.4	3.0 ± 0.1
Triacylglycerols	21.4 ± 3.1	23.6 ± 0.6	29.0 ± 6.9	24.4 ± 3.4
Free Fatty Acids	5.7 ± 0.4	18.0 ± 4.4	6.1 ± 0.6	13.8 ± 2.8
Alcohols	2.1 ± 3.7	—	—	3.4 ± 1.6
Sterols	6.9 ± 1.4	5.6 ± 0.5	2.7 ± 2.3	3.1 ± 2.0
Diacylglycerols	1.1 ± 0.5	—	—	—
Acetone-Mobile Polar Lipids	17.4 ± 3.3	30.9 ± 5.0	19.9 ± 2.3	28.5 ± 1.6
Phospholipids	23.8 ± 5.7	13.4 ± 0.3	29.4 ± 4.8	20.3 ± 4.6
Total	100 %	100 %	100 %	100 %

Table 5.2 Lipid class composition of senescent cultures of *P. pungens* and *P. multiseries*. Results are reported as percent of total lipids detected (mean ± standard deviation, n=3-4).

	P. multiseries		*P. pungens*	
Lipid Class	Treated	Untreated	Treated	Untreated
Hydrocarbons	7.7 ± 4.5	9.8 ± 3.5	6.9 ± 5.5	8.5 ± 5.0
Steryl/Wax Esters	1.2 ± 2.1	0.6 ± 0.1	0.8 ± 0.3	0.3 ± 0.4
Methyl Esters	—	—	0.1 ± 0.1	—
Ethyl Ketones	1.0 ± 1.0	0.8 ± 1.5	0.6 ± 0.9	0.2 ± 0.1
Methyl Ketones	—	0.3 ± 0.5	—	—
Triacylglycerols	7.0 ± 5.6	1.0 ± 1.2	12.2 ± 1.6	0.6 ± 0.9
Free Fatty Acids	9.4 ± 3.2	19.7 ± 1.4	12.1 ± 2.5	26.7 ± 6.0
Alcohols	—	1.1 ± 0.5	1.4 ± 1.2	0.6 ± 0.5
Sterols	5.9 ± 1.6	8.4 ± 2.0	5.6 ± 2.1	7.5 ± 4.7
Diacylglycerols	1.8 ± 2.3	5.4 ± 4.0	2.0 ± 0.4	6.7 ± 3.5
Acetone-Mobile Polar Lipids	26.5 ± 2.8	26.4 ± 3.0	22.4 ± 1.2	25.6 ± 5.0
Phospholipids	39.4 ± 1.7	26.6 ± 1.7	36.0 ± 3.1	23.3 ± 1.1
Total	100 %	100 %	100 %	100 %

studies (Volkman *et al*, 1989; Parrish *et al*, 1991; Dunstan *et al*, 1994) may be artefactual.

The senescent cultures (Table 5.2) harvested a month later displayed a different pattern among treated and untreated samples. A decrease in FFA proportions and an almost equivalent increase in PL with boiling water treatment was still apparent, but these samples did not contain a significantly different proportion of AMPL. In addition, a very substantial increase in TAG was observed in the treated samples. These senescent cultures were unhealthy with spore formation starting to occur, and significant levels of bacteria were present with cultures containing 25 - 30% detritus. Presumably the boiling water deactivated any lipases associated with intact diatoms, but, in unhealthy cultures such as these, autolysis and the resulting bacterial growth would be expected to generate large amounts of FFA. It is likely that bacteria made a significant contribution to the lipid data, partially explaining the differences in composition in the stationary phase and senescent cultures. The difference in growth phase may also be a factor.

A FFA content of 25% has previously been suggested as a reasonable value to use as a maximum proportion normally produced by algal cells in seawater (Parrish, 1988). It was also recommended that higher values be taken as an indicator of *in situ* degradation. However, fatty acids tend not to exist as free carboxylic acids because they may interfere with enzyme function (Gurr and Harwood, 1991), so reports of high levels of FFA are likely artefacts due to cell damage. In this study, precautions were taken to deactivate lipases, but a FFA level of approximately 6% was still obtained in the healthy stationary phase cultures. It seems that levels of FFA near 10% of total lipids would be a more appropriate maximal

value to accept in lipid results. Values above this level would indicate poor sample handling techniques unless significant degradation due to bacteria was a possibility, and fatty acid bacterial markers could be used to evaluate their influence (see Section 5.3.3). Obtaining FFA percentages near zero may not be a reasonable expectation in routine lipid analyses and, in degraded algal detritus, proportions as high as 15% may occur naturally (Table 5.2).

5.3.2 Lipid Class Results

Treated early stationary phase *P. multiseries* samples (Table 5.1) contained significantly different ($P<0.05$) proportions of TAG, AMPL and PL than the treated senescent culture (Table 5.2), while the treated early stationary phase *P. pungens* culture contained significantly different ($P<0.05$) amounts of TAG, DAG and FFA than the treated senescent culture. Senescent cultures are particularly susceptible to autolysis, and the resulting cell exudates commonly support extensive bacterial growth (Parsons *et al*, 1984). In this case, it is likely that bacteria were involved in lipid breakdown, as well as making a contribution to the biomass. Because of this bacterial growth in the senescent cultures, the focus will be on lipid class and fatty acid composition of cells harvested in early stationary phase. Boiling water treatment was effective in deactivating lipases and, in light of this, only early stationary phase samples treated with boiling water will be discussed in terms of lipid class composition.

The amount of total lipid per cell varied significantly ($P<0.05$) between *P. multiseries* and *P. pungens* with *P. pungens* containing approximately two times the amount of lipid per cell. The concentrations of 2.7 ± 0.4 and 6.5 ± 0.7 pg cell^{-1} in *P. multiseries* and *P. pungens*,

respectively, are at the low end of the range of 2.6 to 67 pg cell^{-1} reported in the literature for pennate diatoms (Parrish and Wangersky, 1987; Parrish *et al*, 1991; Dunstan *et al*, 1994; Renaud *et al*, 1995). Qualitatively, the lipid class proportions were quite similar in *P. multiseries* and *P. pungens* after treatment with boiling water, but some differences are notable. For example, proportions of SE/WE, ST and PL were significantly different ($P<0.05$) in the two cultures and, generally, *P. pungens* contained larger percentages of TAG, AMPL and PL. The similarities in lipid class proportions reflect the taxonomic similarities of the two organisms, while differences in amounts of lipids per cell may indicate that conditions were not identical in the two culture media.

The lipid class composition of stationary phase *P. multiseries* has previously been reported, but very different results were obtained (Parrish *et al*, 1991). Most noticeably, FFA were found to comprise 40% of total lipids, with TAG and PL comprising only 2.1 and 6.7%, respectively. Such high levels of FFA suggest extensive degradation of acyl lipids which is also the likely cause of the small proportions of TAG and PL found in that study. Attempts to deactivate lipolytic enzymes were made in that study by treating the cells with isopropyl alcohol (Christie, 1989) and by fast freezing in liquid nitrogen, but high levels of FFA persisted. Since boiling water treatment was not employed at that time, comparisons between those results (Parrish *et al*, 1991) and the current study may be difficult.

5.3.3 Fatty Acid Composition

The fatty acid compositions (Tables 5.3 and 5.4) of all four samples were typical of diatoms (Volkman *et al*, 1989; Viso and Marty, 1993; Dunstan *et al*, 1994) with 14:0, 16:1n-7, 16:4n-1 and 20:5n-3 among the most abundant fatty acids. High ratios of 16:1/16:0 and ΣC16/ΣC18 are also characteristic of diatoms, and elevated levels, as compared to other microalgal classes, were obtained for these cultures (Tables 5.3 and 5.4). Several reports of fatty acid compositions of the related *Nitzschia* species are available in the literature (Nichols *et al*, 1986; Parrish *et al*, 1991; Viso and Marty, 1993; Dunstan *et al*, 1994) and are generally similar to the results obtained here with the fatty acids mentioned above predominating. The senescent cultures contained high levels of 18:1n-7 (> 5% of total fatty acids). Such levels are not expected in diatoms and also serve to indicate substantial bacterial growth in these senescent cultures, as this fatty acid has been employed as a bacterial indicator (Perry *et al*, 1979; Wakeham and Beier, 1991).

The fatty acid composition of these treated cultures was also examined to identify any fatty acid or fatty acid ratio that could be employed as a signature compound for toxic *P. multiseries*. The fatty acid 16:4n-1 is typically found in diatoms at levels of 1-2% of total fatty acids but was present in this study at proportions up to about 7.5% in both *P. pungens* and *P. multiseries*. This fatty acid has been proposed as a marker for toxic *P. multiseries* (Parrish *et al*, 1991), but clearly, with similar levels produced by *P. pungens*, it would not be possible to distinguish these two species. However, with elevated amounts of that acid present in both cultures, it may be possible to use 16:4n-1 to differentiate *Pseudo-nitzschia* species from

Table 5.3 Fatty acid composition of early stationary phase cultures of *P. pungens* and *P. multiseries*. Results are reported as percent of total fatty acids (mean ± standard deviation, n=3).

	P. multiseries		*P. pungens*	
Fatty Acid	Treated	Untreated	Treated	Untreated
Branched				
i-15:0	0.18 ± 0.05	0.12 ± 0.11	0.46 ± 0.03	0.48 ± 0.02
ai-15:0	—	—	0.36 ± 0.04	0.59 ± 0.33
i-17:0	0.75 ± 0.10	0.77 ± 0.03	0.52 ± 0.09	0.39 ± 0.11
Subtotal	0.93 ± 0.14	0.89 ± 0.09	1.35 ± 0.14	1.46 ± 0.22
Saturated				
14:0	12.20 ± 0.49	13.73 ± 0.40	13.3 ± 1.0	12.64 ± 0.27
15:0	0.59 ± 0.08	0.52 ± 0.02	0.41 ± 0.03	0.33 ± 0.02
16:0	5.05 ± 0.41	5.15 ± 0.34	2.99 ± 0.09	2.71 ± 0.57
18:0	0.06 ± 0.10	—	—	0.38 ± 0.22
22:0	—	—	0.05 ± 0.08	—
24:0	1.70 ± 0.17	1.61 ± 0.05	3.70 ± 0.34	3.4 ± 1.0
Subtotal	19.60 ± 0.57	21.00 ± 0.27	20.4 ± 1.3	19.5 ± 2.1
Monounsaturated				
16:1n-9	—	—	0.26 ± 0.44	0.21 ± 0.37
16:1n-7	23.25 ± 0.82	25.3 ± 1.0	22.80 ± 0.45	25.10 ± 0.36
16:1n-5	1.43 ± 0.03	1.58 ± 0.03	1.90 ± 0.03	2.00 ± 0.02
18:1n-9	1.30 ± 0.23	1.13 ± 0.16	0.80 ± 0.23	0.29 ± 0.13
18:1n-7	3.16 ± 0.24	2.84 ± 0.19	1.21 ± 0.10	1.58 ± 0.48
18:1n-5	0.43 ± 0.07	0.39 ± 0.03	0.40 ± 0.02	0.44 ± 0.08
20:1n-11	0.21 ± 0.36	0.07 ± 0.12	0.24 ± 0.11	0.08 ± 0.08
20:1n-9	—	—	0.08 ± 0.07	0.07 ± 0.06
Subtotal	29.78 ± 0.28	31.27 ± 0.99	27.68 ± 0.17	29.78 ± 0.67
Polyunsaturated				
16:2n-7	3.64 ± 0.18	3.97 ± 0.07	3.49 ± 0.19	3.86 ± 0.09
16:2n-4	4.84 ± 0.28	4.93 ± 0.13	4.20 ± 0.16	3.99 ± 0.07
16:3n-4	2.35 ± 0.12	2.55 ± 0.08	1.79 ± 0.09	1.53 ± 0.06
16:4n-1	6.87 ± 0.33	7.41 ± 0.20	7.69 ± 0.31	6.04 ± 0.42
18:2n-6	0.37 ± 0.04	0.39 ± 0.03	0.16 ± 0.01	0.17 ± 0.02
18:3n-6	0.27 ± 0.07	0.37 ± 0.02	0.25 ± 0.02	0.30 ± 0.02
18:4n-3	0.65 ± 0.04	0.73 ± 0.00	0.13 ± 0.00	0.15 ± 0.02
20:2n-6	0.08 ± 0.13	—	—	—
20:3n-6	—	—	0.04 ± 0.06	—
20:4n-6	0.29 ± 0.02	0.33 ± 0.03	0.59 ± 0.02	0.61 ± 0.06
20:4n-3	0.08 ± 0.07	0.09 ± 0.08	—	—
20:5n-3	27.3 ± 1.8	24.3 ± 1.0	30.79 ± 0.87	31.5 ± 2.2
Subtotal	46.7 ± 2.6	45.07 ± 0.54	49.1 ± 1.6	48.1 ± 2.7
Total	97.1 ± 2.5	98.24 ± 0.37	98.55 ± 0.19	98.82 ± 0.26
16:1/16:0	4.91 ± 0.46	5.24 ± 0.52	8.34 ± 0.37	10.3 ± 2.0
ΣC16/ΣC18	7.66 ± 0.75	8.69 ± 0.23	15.4 ± 1.8	14.5 ± 3.9

Table 5.4 Fatty acid composition of senescent cultures of *P. pungens* and *P. multiseries*. Results are reported as percent of total fatty acids (mean ± standard deviation, n=3-4).

	P. multiseries		*P. pungens*	
Fatty Acid	Treated	Untreated	Treated	Untreated
Branched				
i-15:0	0.17 ± 0.16	0.19 ± 0.04	1.38 ± 0.22	1.46 ± 0.11
ai-15:0	—	—	1.25 ± 0.24	1.37 ± 0.12
i-17:0	0.77 ± 0.04	0.65 ± 0.02	0.74 ± 0.10	0.58 ± 0.07
Subtotal	0.94 ± 0.18	0.84 ± 0.05	3.37 ± 0.55	3.40 ± 0.26
Saturated				
14:0	11.81 ± 0.62	13.80 ± 0.38	13.6 ± 1.4	14.48 ± 0.38
15:0	0.46 ± 0.03	0.54 ± 0.05	0.40 ± 0.04	0.40 ± 0.02
16:0	7.46 ± 0.13	8.13 ± 0.40	3.76 ± 0.34	3.43 ± 0.25
18:0	1.03 ± 0.06	0.92 ± 0.18	0.57 ± 0.16	0.51 ± 0.05
24:0	2.11 ± 0.04	1.88 ± 0.13	4.87 ± 0.72	4.47 ± 0.27
Subtotal	22.86 ± 0.71	25.27 ± 0.70	23.2 ± 1.7	23.29 ± 0.17
Monounsaturated				
16:1n-9	1.05 ± 0.14	0.89 ± 0.28	0.73 ± 0.49	0.92 ± 0.10
16:1n-7	19.07 ± 0.81	25.9 ± 1.5	19.97 ± 0.60	23.77 ± 0.87
16:1n-5	1.18 ± 0.02	1.39 ± 0.03	1.88 ± 0.10	2.14 ± 0.07
18:1n-9	2.42 ± 0.23	2.37 ± 0.10	1.07 ± 0.53	0.81 ± 0.06
18:1n-7	19.4 ± 1.5	16.66 ± 0.89	4.99 ± 0.69	5.23 ± 0.43
18:1n-5	1.52 ± 0.12	1.26 ± 0.10	0.60 ± 0.10	0.64 ± 0.03
20:1n-11	—	—	0.09 ± 0.18	0.05 ± 0.11
20:1n-9	—	—	0.04 ± 0.08	—
Subtotal	44.6 ± 1.2	48.5 ± 1.1	29.4 ± 1.5	33.56 ± 0.82
Polyunsaturated				
16:2n-7	1.32 ± 0.09	1.74 ± 0.12	2.32 ± 0.19	2.61 ± 0.13
16:2n-4	2.79 ± 0.12	3.29 ± 0.16	3.02 ± 0.18	3.02 ± 0.09
16:3n-4	0.98 ± 0.10	1.23 ± 0.10	1.20 ± 0.13	*0.88 ± 0.05
16:4n-1	4.63 ± 0.45	5.57 ± 0.33	7.22 ± 0.35	4.63 ± 0.33
18:2n-6	0.48 ± 0.08	0.52 ± 0.11	0.32 ± 0.12	0.30 ± 0.04
18:2n-4	0.43 ± 0.13	0.35 ± 0.11	0.21 ± 0.16	0.15 ± 0.04
18:3n-6	0.21 ± 0.02	0.23 ± 0.03	0.28 ± 0.04	0.39 ± 0.02
18:4n-3	0.41 ± 0.02	0.46 ± 0.03	0.13 ± 0.09	0.16 ± 0.01
20:4n-6	0.43 ± 0.01	0.34 ± 0.02	0.62 ± 0.03	0.66 ± 0.01
20:4n-3	0.17 ± 0.01	0.20 ± 0.04	—	—
20:5n3	18.3 ± 1.0	9.9 ± 1.6	26.2 ± 3.3	24.68 ± 0.57
Subtotal	30.1 ± 1.7	23.9 ± 1.1	41.5 ± 3.6	37.48 ± 0.81
Total	98.53 ± 0.23	98.42 ± 0.42	97.40 ± 0.27	97.72 ± 0.23
16:1/16:0	2.86 ± 0.15	3.47 ± 0.24	6.04 ± 0.55	7.87 ± 0.76
ΣC16/ΣC18	1.50 ± 0.16	2.12 ± 0.16	5.02 ± 0.91	5.08 ± 0.38

other diatoms. In addition, the general diatom marker, ΣC16/ΣC18, did show consistent variation among cultures of *P. multiseries* and *P. pungens*. This ratio was significantly higher ($P<0.05$) by a factor of approximately two in cultures of *P. pungens* due to the lower proportions of 18:1n-9 and 18:1n-7 in that culture. In the laboratory, this ratio could be applied as a biomarker to differentiate cultures of the two species. There were also significant differences ($P<0.05$) in the proportions of other fatty acids, including 16:0, 16:3n-4, 18:1n-7 and 24:0, in the two cultures, but these acids are commonly found in many classes of microalgae and would not be useful in detecting *P. multiseries* in a field situation. Application of the ratio ΣC16/ΣC18 as a biomarker in the field would also be problematic, as elevated levels of this ratio are characteristic of almost all diatoms. In natural samples, perhaps some other class of compounds would be more useful as biomarkers. Bacillariolides, which are oxygenated lipids thought to be derived from 20:5n-3 (Gerwick, 1994), are one such group (Wang *et al*, 1993).

In a secondary study (Table 5.5), the fatty acid composition of another strain of *P. multiseries*, KP105, was compared to that of the strain discussed above, CCMP 1660. The proportions of several major acids fluctuated widely among the two strains. Similar variability among clones grown under identical culture conditions has been observed in the fatty acids of *Isochrysis galbana* (Alonso *et al*, 1992). These fluctuations in proportions also raise some doubt concerning the usefulness of fatty acid biomarkers for the identification of specific species. However, proportions of major fatty acids in stationary and senescent phases of both strains did vary consistently with decreased proportions of all fatty acids in Table 5.5, with

Table 5.5 Variation in the proportions of major fatty acids in two strains of *P. multiseries* in stationary and senescent growth phases. Both strains were treated with boiling water prior to extraction.

	P. multiseries					
	KP105			CCMP 1660		
	Stationary	Senescent	% Change	Stationary	Senescent	% Change
14:0	18.0	16.0	-10.7	12.2	11.8	-3.2
16:0	4.5	6.9	+53.1	5.1	7.5	+47.7
16:1n-7	28.9	15.5	-46.3	23.3	19.1	-18.0
16:2n-4	5.1	2.4	-52.0	4.8	2.8	-42.4
16:3n-4	5.2	3.3	-37.4	2.4	1.0	-58.3
16:4n-1	4.7	4.3	-7.9	6.9	4.6	-32.6
20:5n-3	21.8	14.8	-32.0	27.3	18.3	-33.0

the exception of 16:0, in the senescent cultures. This suggests that, despite the extensive bacterial growth, there is consistency in the biochemical alterations of fatty acid proportions in response to stress in the two strains.

As with the lipid class composition, the fatty acid compositions were also different in cultures with and without boiling water treatment. In general, boiling water treatment resulted in a larger proportion of PUFA and a smaller proportion of monounsaturated fatty acids. This suggests that, in addition to the deactivation of lipolytic enzymes, some mechanism of PUFA degradation was also inhibited. It is possible that labile PUFA were more prone to exposure to oxygen or light as FFA, leading to autoxidation, rather than when esterified in acyl lipids. Boiling water treatment resulted in a higher proportion of esterified fatty acids that were protected from degradation by incorporation in tissues and membranes. A second explanation involves the lipoxygenase enzyme commonly found in diatoms. These lipoxygenases insert a hydroperoxide functionality on the methylene group between double bonds in FFA and initiate breakdown of PUFA. In untreated samples, lipases may have produced a larger proportion of FFA on which lipoxygenases could act, resulting in less PUFA than in treated samples. It is also likely that boiling water treatment is capable of deactivating lipoxygenases as well as lipases. Whatever the explanation, PUFA levels were higher in treated samples, suggesting that, in addition to questionable reports of high FFA proportions in the literature, PUFA levels in diatoms may also be higher than currently thought. As previously indicated for *Skeletonema costatum* (Berge *et al*, 1995), treatment of all phytoplankton samples,

particularly diatoms, with boiling water to deactivate any lipolytic enzymes would seem to be a good practice.

5.4 Conclusions

Lipid class and fatty acid compositions of stationary phase cultures of the toxic diatoms *Pseudo-nitzschia multiseries* and *P. pungens* were determined. The lipid class compositions of both diatoms were similar, with PL, TAG and AMPL as the major classes. Both pennate diatoms also displayed similar fatty acid compositions that were typical of diatoms, with high levels of 14:0, 16:1n-7, 16:2n-4 and 20:5n-3. In addition, elevated levels (>7%) of the fatty acid 16:4n-1 were found in both species. This fatty acid can be used as a general diatom marker (Chapters 4 and 6) but also has potential as a signature compound in differentiating *Pseudo-nitzschia* species from other diatoms. The taxonomic similarities of the diatoms were apparent in the very similar lipid class and fatty acid compositions. Treatment of cells with boiling water was effective in deactivating lipolytic enzymes and resulted in a significant decrease ($P<0.05$) in FFA, a breakdown indicator, and a general increase in PUFA in all samples. Thus, the routine use of boiling water to deactivate lipolytic enzymes in diatom samples is recommended.

Chapter 6 - FATTY ACID COMPOSITION OF PHYTOPLANKTON, SETTLING PARTICULATE MATTER AND SEDIMENTS AT A SHELTERED BIVALVE AQUACULTURE SITE

6.1 Introduction

In Chapter 4, the biogeochemistry of the deep, fjord-like Trinity Bay was investigated to assess carbon cycling and determine sources and fates of organic material. Here, a similar type of study, examining the fatty acid composition of phytoplankton, settling particulate matter, sediments and bivalves in 1995 and 1996, is applied to a very different environment. Pilley's Tickle is a shallow, sheltered inlet located in Notre Dame Bay in northern Newfoundland containing several blue mussel (*Mytelis edulis*) aquaculture sites. Determination of the quality of lipid material in this environment is particularly important with large numbers of bivalves depending on that matter for growth. In addition to studying the biogeochemistry, this environment provides an opportunity to compare the fatty acid composition of phytoplankton with the changing requirements of bivalves in different growth stages.

The particular site examined in this study, Barred Island Cove, was chosen because it has been permanently closed due to the presence of cysts of the paralytic shellfish poison (PSP) producer, *Alexandrium fundyense* (Schwinghamer *et al*, 1994) and it was hoped that a fatty acid biomarker could be determined for that toxic dinoflagellate. Non-toxic cysts of another dinoflagellate, *Scrippsiella trochoidea*, that were similar in appearance, were also

found in this area. To identify a biomarker to differentiate between the two dinoflagellates, the fatty acid composition of vegetative cultures of toxic *A. fundyense* and non-toxic *S. trochoidea* was determined.

The use of particular fatty acids, groups of fatty acids and fatty acid ratios as biomarkers of various sources has been described in Sections 4.1 and 4.3.1. Those same markers were also applied here, along with the following additional biomarkers: 18:5n-3 for dinoflagellates and long-chain saturates for terrestrial material. The fatty acid 18:5n-3 has been reported in numerous flagellates, in particular dinoflagellates (Harvey *et al*, 1988; Volkman *et al*, 1989; Dunstan *et al*, 1992; Viso and Marty, 1993; Parrish *et al*, 1994), and increasing proportions of this fatty acid reflect the increasing importance of dinoflagellates as sources of organic material. Long-chain fatty acids such as 20:0, 22:0 and 24:0, have been applied as markers of terrestrial plants in marine sediments (Harvey, 1994; Santos *et al*, 1994; Colombo *et al*, 1997) and are used as such here. Floristic examinations of the samples also allowed the suitability of the phytoplankton markers to be evaluated. Finally, the use of stable carbon isotope analysis to determine carbon sources and biochemical pathways was investigated by application to a net-tow and a terrestrial plant sample.

6.2 Experimental

Vertical net-tow samples were collected several times in Barred Island Cove in Pilley's Tickle in Notre Dame Bay, NF, in summer and fall of 1995 and 1996 (Figure 6.1). Net-tows (20 μm mesh) were taken vertically from a depth of approximately 10 m (near bottom)

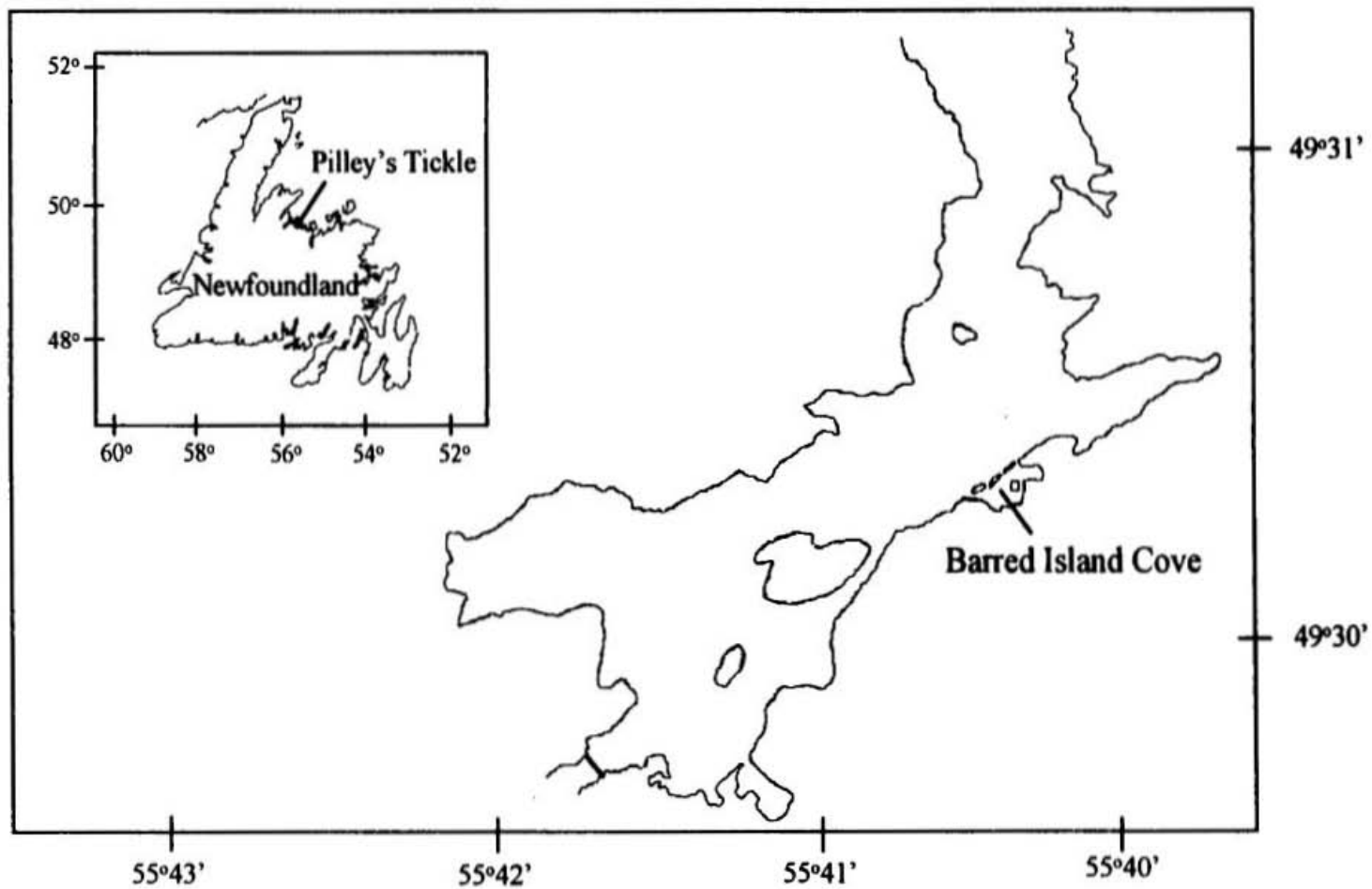


Figure 6.1 Map of Barred Island Cove and Pilley's Tickle.

to the surface and the collected plankton was stored in bottles on ice. Lipid samples were taken by resuspending the plankton and filtering on glass-fibre (GF/C) filters. Sediment traps consisting of four PVC tubes were deployed 2-3 m from the surface (total depth of 10 m) for 30 days in August and November of 1995 and for one day in October of 1996. As in Trinity Bay, one litre of 40 ppt NaCl solution was added to each trap and the remainder was filled with seawater before deployment. On recovery, particles were allowed to settle for 30 minutes before collection. Particles were then resuspended and filtered for lipid analysis. SCUBA divers collected bottom sediment cores by hand in modified 50 ml centrifuge tubes in 1995 from locations directly below socks containing blue mussels. These sediments were originally collected to determine concentrations of cysts of *A. fundyense* so they were stored in seawater at 0 °C until extraction. Mussel samples were taken from the socks deployed in Barred Island Cove. Lipids were extracted from all samples with the modified Folch *et al* (1957) method outlined in Section 3.2.3.1. Methyl esters of fatty acids were formed (Section 3.2.6.1) and determined as described in Section 3.2.10.1.

GC-C-IRMS was performed using a VG Isochrom II system equipped with a Hewlett Packard 5890 GC. FAME were separated using the conditions described in Section 3.2.10.1. The $\delta^{13}C$ values of the CO_2 produced by combustion of FAME were measured by GC-C-IRMS relative to the Peedee Belemnite standard and reported according to the equation given in Section 2.4.2.6. The $\delta^{13}C$ values were then corrected for the isotopic composition of the methyl ester carbon added during esterification (Abrajano *et al*, 1994).

Cultures of vegetative cells of *Alexandrium fundyense* and *Scrippsiella trochoidea* were obtained from the National Research Council of Canada (Halifax). Both cultures were grown in Guillard's medium L1 (Guillard and Hargraves, 1993) at 16 °C. The average light intensity was 150 μmol photons m^{-1} s^{-1} with a 14:10 light:dark cycle. *A. fundyense* was harvested in late logarithmic phase while *S. trochoidea* was harvested in early logarithmic phase. Both cultures were filtered on glass fibre filters (GF/C) and were extracted with the modified Folch *et al* (1957) method. Two other species of *Alexandrium* were cultured from cysts recovered from Barred Island Cove sediments. Both of these species were grown at the Ocean Science Centre in L1 media with the same light:dark cycle and temperature as above.

6.3 Results and Discussion

6.3.1 Net-Tows

The fatty acid compositions of the net-tow samples fluctuated with sampling period in both 1995 and 1996 (Figure 6.2, Appendices II and III). The large proportion of PUFA in all samples (>30%), particularly 20:5n-3 and 22:6n-3, reflected the contribution of marine phytoplankton. In addition, variations in phytoplankton composition corresponded to changes in specific fatty acid markers. For example, microscopic analysis of algal samples (Figure 6.3a) revealed that in late August 1995 a diatom bloom of *Skeletonema costatum* (C. McKenzie, pers. comm.) had occurred and this was reflected in the diatom markers where all values were elevated on those dates (Figure 6.4a). Following this, in October, the dinoflagellates *Ceratium fuses* and *Scrippsiella trochoidea* were the dominate organisms with

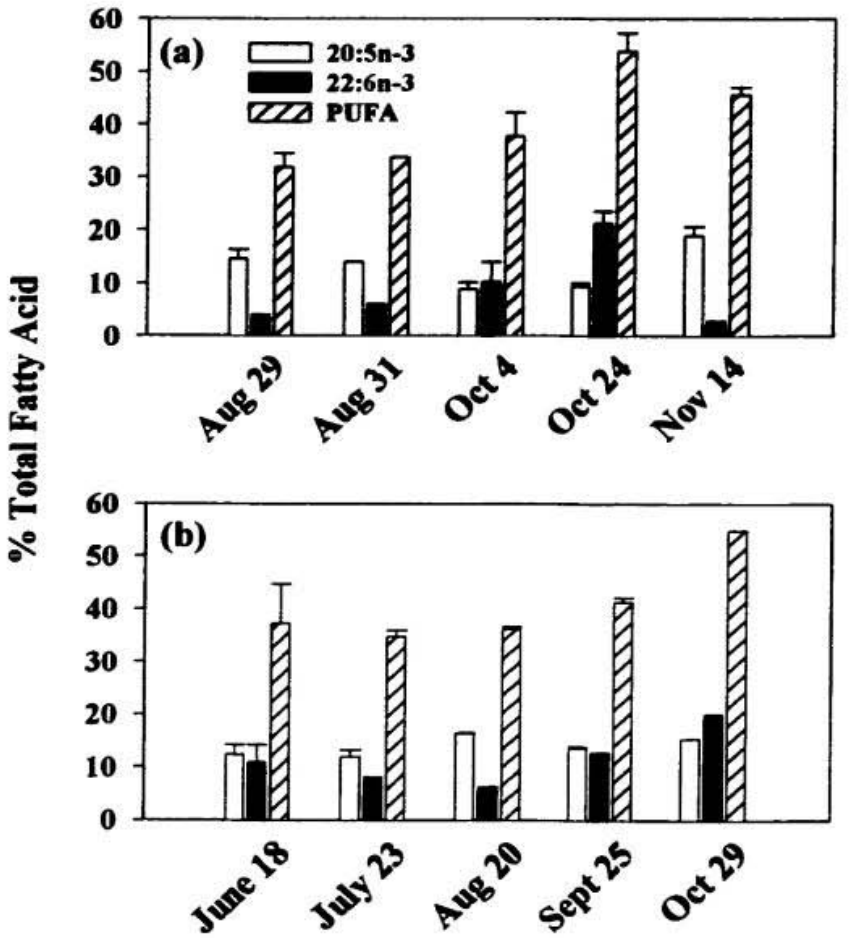


Figure 6.2 Proportions of 20:5n-3, 22:6n-3 and PUFA as percent of total fatty acids in net-tows collected in (a) 1995 and (b) 1996 (mean ± standard deviation, n = 1-3).

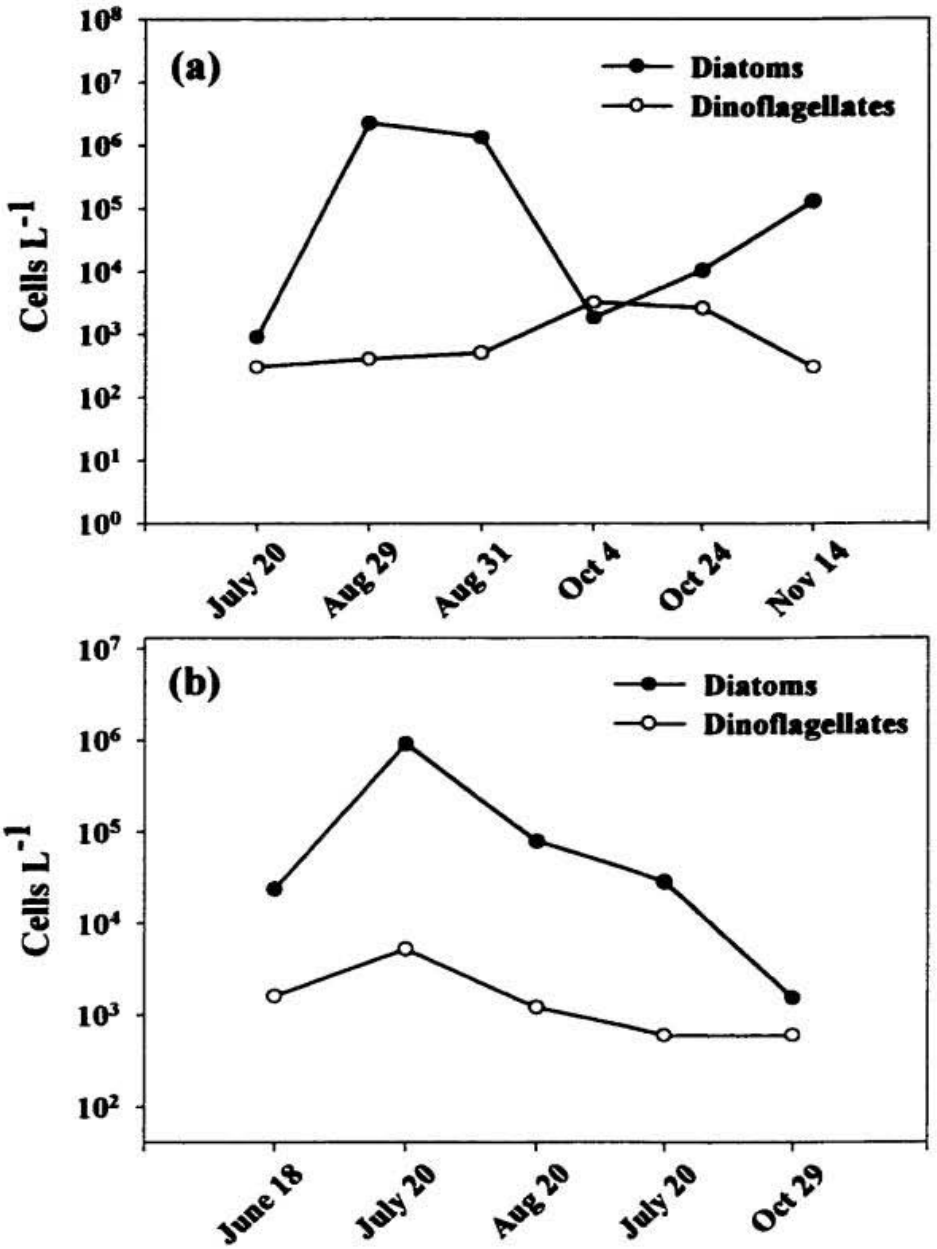


Figure 6.3 Fluctuations in phytoplankton composition with sampling date in Barred Island Cove in (a) 1995 and (b) 1996.

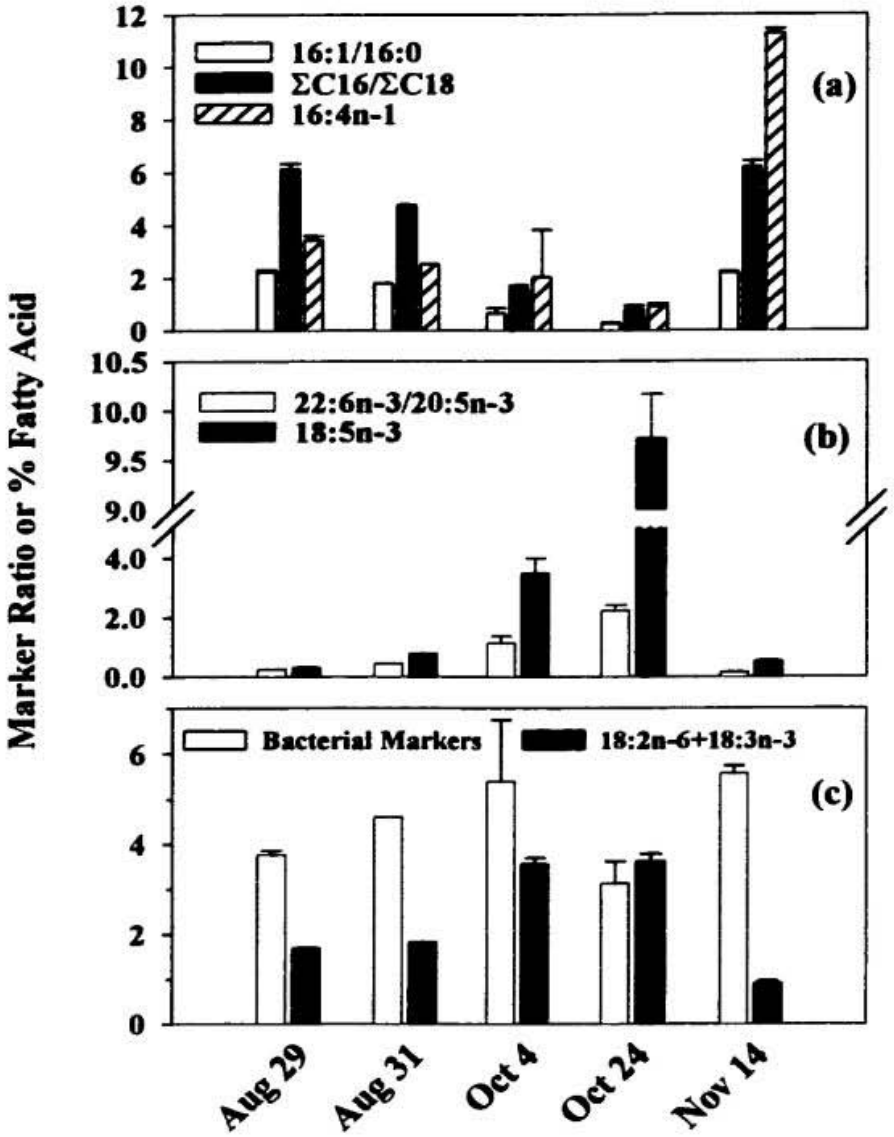


Figure 6.4 Values of markers in net-tow samples collected in 1995 (mean ± standard deviation, n = 1 - 3). (a) Diatom markers; (b) dinoflagellate markers and (c) bacterial (15:0 + 17:0 + branched chain fatty acids) and terrestrial (18:2n-6 + 18:3n-3) markers.

blooms of both occurring at the end of that month. Again, this was evident in the dinoflagellate markers, with those markers increasing in value at the beginning of October and reaching a maximum in late October (Figure 6.4b). Finally, in late November, a bloom of *Chaetoceros* sp. occurred and this was clearly represented by the diatom markers where elevated values of all three markers were obtained and a maximum in 16:4n-1 was reached (Figure 6.4a).

In 1996, levels of the diatom markers also corresponded to fluctuations in plankton species, with the highest diatom marker values in July and August when *S. costatum*, *Chaetoceros* sp. and *Nitzschia closterium* were abundant (Figure 6.5a). However, the dinoflagellate biomarkers do not correspond so well (Figure 6.5b). The dinoflagellate concentrations fluctuate between approximately 500 and 1500 cell L^{-1} with a maximum in July (Figure 6.3b). This maximum was not seen in Figure 6.5b because it coincides with the diatom bloom. This diatom bloom involves much larger concentrations of cells which produce elevated levels of diatom fatty acids. These diatom markers mask the dinoflagellate markers, particularly 22:6n-3/20:5n-3 and this is an inherent problem in the use of marker ratios. In fact, it is likely that this masking of the dinoflagellate signal occurs throughout the 1996 sampling period because at all sampling dates, except October 29, diatoms were present in much larger numbers than dinoflagellates.

It was assumed that the net-tows were collecting fresh, marine plankton so it was surprising to find elevated levels of bacterial (branched and odd carbon-numbered fatty acids) and terrestrial plant biomarkers (18:2n-6 and 18:3n-3) in those samples (Figures 6.4c and

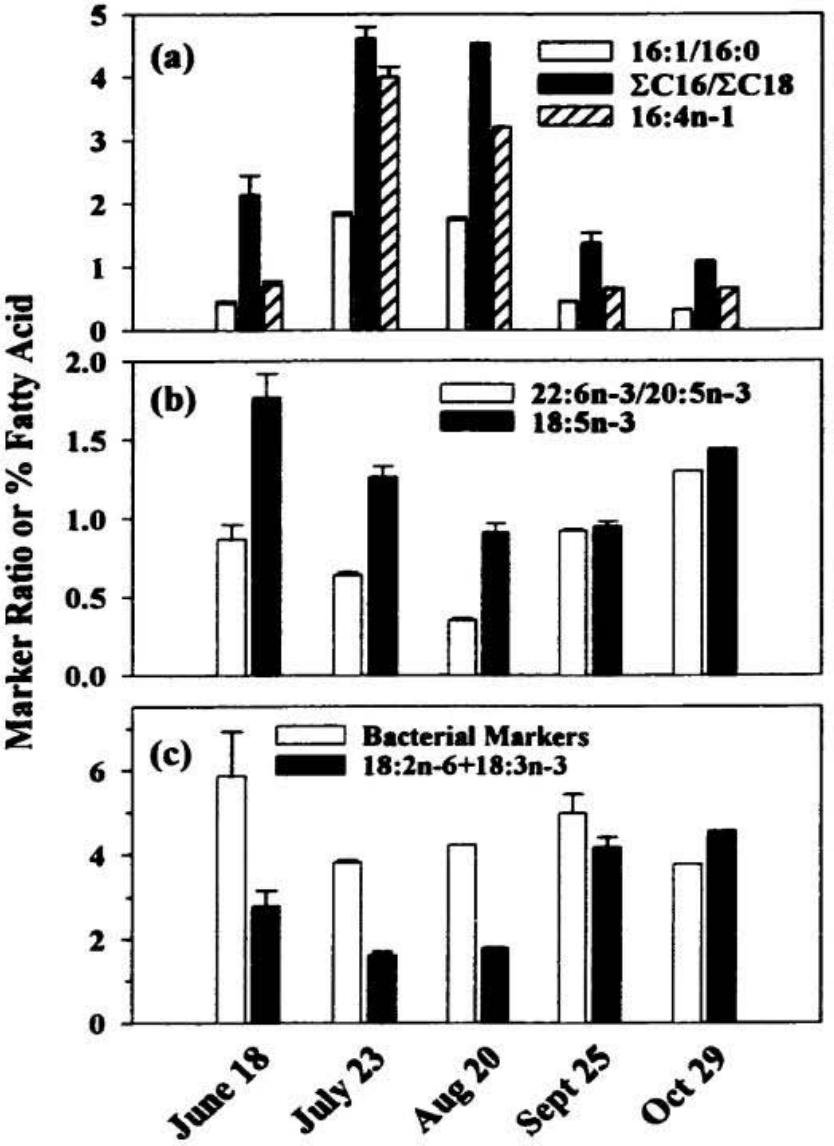


Figure 6.5 Values of markers in net-tow samples collected in 1996 (mean ± standard deviation, n = 1-3). (a) Diatom markers; (b) dinoflagellate markers and (c) bacterial (15:0 + 17:0 + branched chain fatty acids) and terrestrial (18:2n-6 + 18:3n-3) markers.

6.5c). In Trinity Bay, bacterial markers in the net-tow samples were all below 2% of total fatty acids but, in Barred Island Cove, the minimum bacterial marker value was higher at 3% and had a maximum of 6%. These bacterial marker levels were more similar to those encountered in sediment traps in both Barred Island Cove and Trinity Bay (Figure 6.6b) containing somewhat degraded plankton material. The inlet in this study was shallow with a maximum depth of 10 m and wind-driven resuspension of bottom sediments was a common occurence (Schwinghamer *et al*, 1994). It seems likely that the net-tows were actually collecting some degraded material from the sediments, in addition to the fresh plankton material.

Several net-tow samples (June 18, September 25 and October 29 in 1995 and October 4 and 29 in 1996), contained terrestrial markers (18:2n-6 and 18:3n-3) above the threshold level of 2.5% (Section 4.3.1). This cove is surrounded by forest with several streams delivering terrestrial plant material to the area. In this environment, a significant terrestrial plant source of organic material would be expected. However, despite this terrestrial contribution, it should be stressed that with such high PUFA levels, the bulk of the material collected in the net-tows was marine in origin.

6.3.2 Sediment Traps

On average, the sediment traps contained more bacterial fatty acids and less PUFA than the net-tow samples (Figure 6.6). Lower levels of monounsaturated fatty acids were also found in the sediment traps. Material collected in sediment traps is not as fresh as that

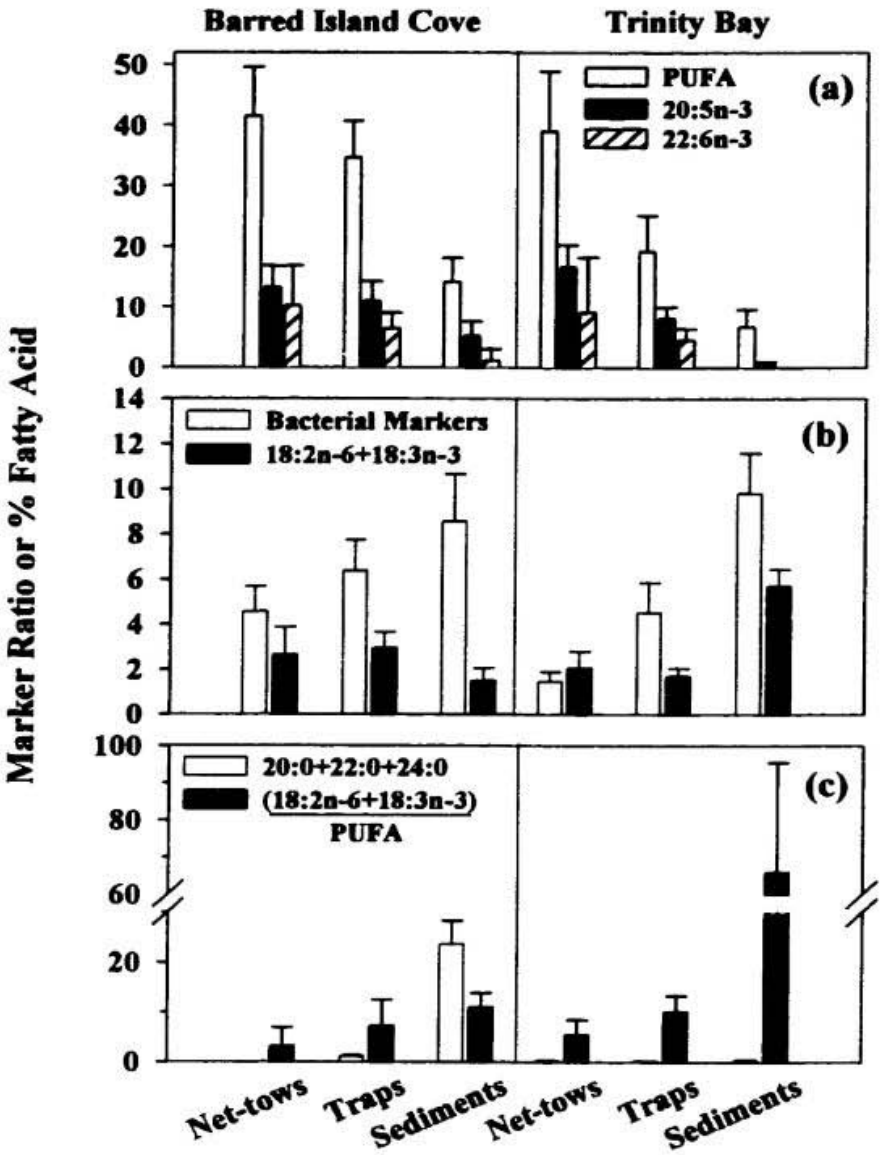


Figure 6.6 Average values of fatty acids and markers in net-tow, sediment trap and sediment samples in Barred Island Cove and Trinity Bay (mean ± standard deviation, n =6-20). (a) PUFA, 20:5n-3 and 22:6n-3 proportions; (b) bacterial (15:0 + 17:0 + branched chain fatty acids) and terrestrial (18:2n-6 + 18:3n-3) markers and (c) sum of 20:0 + 22:0 + 24:0 and terrestrial markers as a proportion of total PUFA.

collected in net-tow samples because sediment traps collect particles that have been settling through the water column and have been exposed to potential degradation by bacteria for longer periods of time. The higher levels of bacterial fatty acids in these samples than in the net-tows support this. This bacterial activity results in lipid breakdown and labile PUFA would be expected to be among the first compounds to be degraded (Meyers and Ishiwatari, 1993; Harvey and Macko, 1997). Indeed, this is reflected in the PUFA proportions in the sediment traps where less PUFA was preserved as compared to the fresh plankton samples. Like the net-tow samples, these sediment traps were collecting resuspended sediments from the floor of the cove and that was likely contributing to the higher levels of bacterial fatty acids and the lower levels of PUFA as well.

Comparisons of sediment trap fatty acids (Appendix IV) and fatty acid biomarkers with corresponding sampling dates in 1995 in the net-tow samples revealed significant differences ($P<0.05$) in almost all parameters considered, including saturated fatty acids, monounsaturated fatty acids, PUFA, phytoplankton markers and terrestrial markers (Figure 6.7). This illustrates the fact that net-tows and sediment traps collect different types of material. Net-tows collect material in the water column over a time span of several minutes and predominantly capture fresh plankton material. The sediment traps in this study, however, were deployed for a minimun of 24 hours and a maximum of 30 days and are expected to collect more degraded material sinking through the water column, as well as fresh plankton material and resuspended sediments. This combination of sources alters the levels of fatty acid proportions in the sediment traps. Trends in fluctuations of fatty acids, however, did

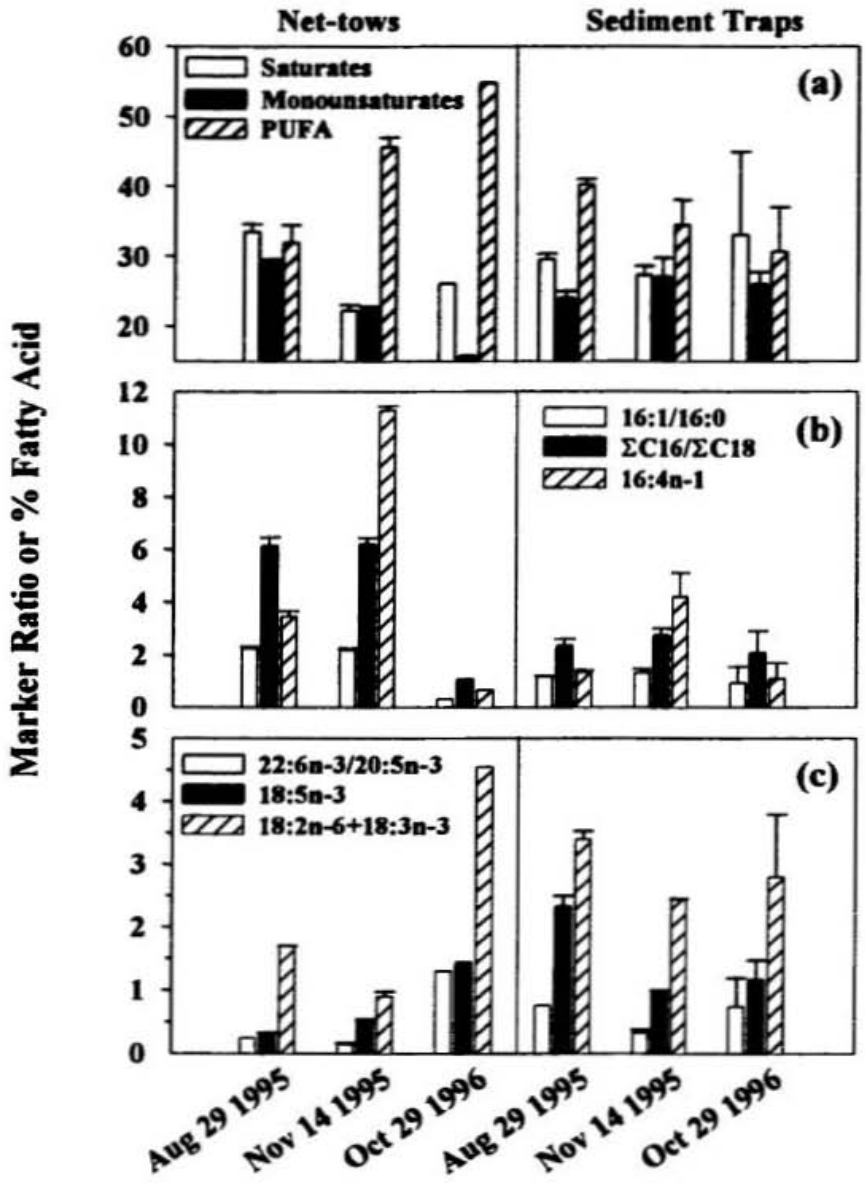


Figure 6.7 Average values of fatty acids and biomarkers in net-tows and sediment traps with common sampling dates (mean ± standard deviation, n=1-4). (a) Total saturated, monounsaturated and polyunsaturated fatty acids; (b) diatom markers and (c) dinoflagellate (22:6n-3/20:5n-3 and 18:5n-3) and terrestrial (18:2n-6 + 18:3n-3) markers.

correspond in sediment traps and net-tows. For example, the diatom marker 16:4n-1 reached a maximum in November in both net-tows and sediment traps. Similar trends were also apparent in the 22:6n-3/20:5n-3 ratio and in the terrestrial markers in net-tows and sediment traps. The variations in fatty acid proportions in tows and traps collected on the same date may serve as a reminder that caution must be exercised when comparing results from different sample types. Similar information was contained in the sediment trap data, but the biomarker signals were masked by contributions from resuspended sediments and bacteria.

6.3.3 Sediments

The fatty acid composition of the sediments (Appendix V) was quite different from that of the net-tow and sediment trap samples, with significantly higher proportions of saturated ($P<0.001$) and bacterial fatty acids ($P=0.022$), and significantly lower ($P<0.001$) PUFA proportions (Figure 6.6). Sediments contain active populations of bacteria which are responsible for the degradation and remineralization of organic material (Deming and Baross, 1993) so higher levels of bacterial markers would be expected. Levels of bacterial markers of 8.6% (Figure 6.6b) in these sediments were quite similar to levels of approximately 10% encountered in sediments from Trinity Bay, NF (Figure 6.6b) and larger than those in the net-tow and sediment trap samples in this study. This suggests that, while resuspension of sediments may be partly responsible for the presence of bacterial markers in the net-tow and sediment trap samples, the bacterial contribution, as indicated by the markers, was greater in the sediments. As discussed in Section 6.3.2, bacterial activity was likely responsible for the

decreased PUFA levels. Increased levels of the more unreactive saturated fatty acids (48%; Appendix V) were also evident as compared to the net-tow samples (28%; Appendices II and III).

The terrestrial markers (18:2n-6 and 18:3n-3) in the sediments, as percent of total fatty acids, were lower than those found in the net-tow and sediment trap samples (Figure 6.6b), seemingly suggesting that terrestrial sources were not important. However, the same terrestrial markers in sediments as a proportion of total PUFA were approximately twice as large on average than those in the net-tow and sediment trap samples (Figure 6.6c). In addition, the long-chain fatty acids 20:0, 22:0 and 24:0, which have been used successfully in the past as terrestrial markers (Harvey, 1994; Santos *et al*, 1994; Colombo *et al*, 1997), comprised approximately 20% of total fatty acids in the sediments (Figure 6.6c), further indicating the importance of preservation of terrestrial material. The presence of 20:5n-3 and 22:6n-3 in the sediments also shows that in addition to terrestrial sources, there still remains a very important phytoplankton source, in agreement with net-tow and sediment trap data.

6.3.4 Stable Carbon Isotopes

Fluctuations in $\delta^{13}C$ of fatty acids generally occur because of differences in carbon source or in biochemistry of organisms. Both marine phytoplankton and most terrestrial plants employ a C3 pathway to metabolize carbon (Libes, 1993) so their biochemistries are similar.

Differences in sources of carbon, however, were clearly reflected in the $\delta^{13}C$ values of an October 24 1995 net-tow sample and a terrestrial plant, the reed, horsetail (*Equisiteum* sp.) (Table 6.1). The terrestrial plant, employing isotopically light atmospheric $CO_{2(g)}$ in photosynthesis, produced fatty acids depleted in ^{13}C with a $\delta^{13}C$ of -33‰ or less. However, the use of isotopically heavier dissolved $CO_{2\ (aq)}$ in photosynthesis in phytoplankton resulted in fatty acids more enriched in ^{13}C. The fatty acids used as terrestrial plant markers, 18:2n-6 and 18:3n-3, had the lightest $\delta^{13}C$ values of -27.6 and -29.4‰, respectively, in the net-tow suggesting that they do, indeed, have a terrestrial source, but the values were not as depleted as those encountered in the terrestrial plant *Equisiteum* sp. It is likely that the slightly enriched $\delta^{13}C$ of 18:2n-6 and 18:3n-3 found in the net tow was a result of those two fatty acids having a small marine phytoplankton contribution as well. Green alga of the classes *Chlorophyceae* and *Prasinophyceae* are known to produce elevated amounts of these fatty acids (up to 50% of total) (Dunstan *et al*, 1992) but, in this area, those types of phytoplankton are rare. Their potential contribution to net-tow samples and influence on terrestrial fatty acids has been taken into consideration by applying a threshold of 2.5 to this marker (Section 4.3.1).

The biomass of the particular net-tow examined isotopically was dominated by dinoflagellates so a large proportion of the fatty acids with similar $\delta^{13}C$ values would be expected to be derived from that source. Chain elongation and desaturation results in depletion of ^{13}C because of the kinetic isotope effect (isotopically lighter fatty acids have lower activation energies and, therefore, react preferentially) (Monson and Hayes, 1980). This, for example, provides an explanation for the slight depletion of 20:5n-3 as compared

Table 6.1 Values of $\delta^{13}C$ of selected fatty acids in a terrestrial plant and a net-tow sample.

	$\delta^{13}C$	
Fatty Acid	*Equisetum* sp.	Net-tow Oct 24 1995
18:2n-6	-33.7	-27.6 ± 0.6
18:3n-3	-33.4	-29.4
18:4n-3	-------	-25.5 ± 0.0
18:5n-3	-------	-17.6 ± 0.8
20:5n-3	-------	-26.7 ± 2.3
22:0	-34.9	-------
22:6n-3	-------	-21.4 ± 0.9
24:0	-34.1	-------

to its precursor 18:4n-3 (see Figure 2.3). If synthesis of 22:6n-3 followed a similar route and proceeded simply through elongation and desaturation of 20:5n-3, a more depleted product would be expected. However, a more enriched 22:6n-3 was present. Synthesis of 22:6n-3 in mammals actually proceeds through a chain shortening step by β-oxidation of 24:6n-3 (Voss *et al*, 1991) and, assuming the same pathway is followed in marine plankton, it is possible that that step resulted in enrichment of ^{13}C. Reverse isotope fractionation can occur if the heavier isotope has a lower activation energy than the lighter isotope (Libes, 1992). The enrichment of 18:5n-3 can be explained if its formation also proceeds through a chain shortening of 20:5n-3.

The above argument does make several assumptions that should be kept in mind: 1) the sample was dominated by dinoflagellates but there was still a contribution from other algae such as diatoms; 2) several species of dinoflagellates were present and there is no guarantee that all dinoflagellates employ the same biochemical pathways; 3) chain shortening may involve a reverse isotope effect but there is little evidence to date in the literature to support this and 4) the $\delta^{13}C$ values of dissolved $CO_{2(aq)}$, which serves as the substrate for fatty acid synthesis, may change over the sampling season (Bieger *et al*, 1997), influencing the $\delta^{13}C$ values of fatty acids. Isotope analysis of pure cultures of dinoflagellates would make the first two assumptions unnecessary and may also shed some light on their biochemistry.

6.3.5 Cultures of Alexandrium fundyense *and* Scrippsiella trochoidea

Cysts of *A. fundyense*, responsible for PSP toxicity in bivalves, were present in the sediments in Barred Island Cove. Non-toxic cysts of another dinoflagellate, *S. trochoidea*, were also found. In order to establish a biomarker for the toxic dinoflagellate, the fatty acid compositions of vegetative cultures of the two organisms were determined. These cultures were grown at the National Research Council (NRC) laboratory in Halifax, NS, under identical conditions of light, growth media and temperature so differences in fatty acid composition should be directly attributable to differences in their biochemistries.

The two cultures exhibited fatty acid compositions (Table 6.2) typical of marine dinoflagellates (Harvey *et al*, 1988; Viso and Marty, 1993; Bodennec *et al*, 1995). The major fatty acids in both cultures, as proportions of total fatty acids and as pg $cell^{-1}$, were 16:0, 18:4n-3, 18:5n-3 and 22:6n-3, and the proportions of saturated fatty acids and PUFA were approximately equivalent. The dinoflagellate markers used in the present study, 22:6n-3/ 20:5n-3 and 18:5n-3, were based in part on the fatty acid composition of these two dinoflagellates.

The fatty acid 18:5n-3 is quite rare and is thought to have a synergistic effect with toxicity (Yasumoto *et al*, 1990) so initially it was a candidate for a marker for *A. fundyense*. It is obvious in the data presented here, however, that the proportion of that fatty acid is larger in *S. trochoidea*, making it inappropriate as a specific marker of *A. fundyense*. Various ratios of 18:5n-3 with other fatty acids were also considered but a marker that differed sufficiently between the two cultures to be relied on in the field was not identified. This similar

Table 6.2 Fatty acid proportions (% total fatty acids) and concentrations (pg cell^{-1}) in *Alexandrium fundyense* and *Scrippsiella trochoidea* cultured at the National Research Council laboratory in Halifax, NS (mean ± standard deviation, n=3).

	A. fundyense		*S. trochoidea*	
Fatty Acid	%	concentration	%	concentration
Branched				
i-15:0	0.76 ± 0.24	1.97 ± 0.81	0.20 ± 0.03	0.28 ± 0.08
ai-15:0	1.00 ± 0.12	2.54 ± 0.42	0.19 ± 0.04	0.26 ± 0.06
Subtotal	1.76 ± 0.25	4.5 ± 1.0	0.39 ± 0.07	0.53 ± 0.14
Saturated				
14:0	10.82 ± 0.46	27.5 ± 2.7	8.3 ± 1.0	11.7 ± 4.1
16:0	22.32 ± 0.20	56.7 ± 5.2	20.7 ± 2.3	29 ± 10
18:0	0.59 ± 0.07	1.50 ± 0.26	1.53 ± 0.13	2.09 ± 0.41
20:0	0 ± 0	0 ± 0	0.67 ± 0.87	0.8 ± 1.0
Subtotal	33.73 ± 0.26	85.7 ± 7.9	31.3 ± 2.4	43.6 ± 14.1
Monounsaturated				
16:1n-7	0.71 ± 0.16	1.83 ± 0.57	1.63 ± 0.20	2.25 ± 0.62
18:1n-9	2.25 ± 0.39	5.69 ± 0.94	6.19 ± 0.24	8.6 ± 2.2
18:1n-7	0.26 ± 0.46	0.7 ± 1.2	2.76 ± 0.72	3.71 ± 0.70
20:1n-9	0 ± 0	0 ± 0	0.40 ± 0.70	0.49 ± 0.85
Subtotal	3.22 ± 0.03	8.19 ± 0.84	11.0 ± 1.4	15.0 ± 2.9
Polyunsaturated				
16:2n-4	0.09 ± 0.02	0.24 ± 0.06	0.18 ± 0.03	0.25 ± 0.02
16:3n-4	0.03 ± 0.05	0.06 ± 0.11	0 ± 0	0 ± 0
18:2n-6	1.28 ± 0.30	3.23 ± 0.53	2.57 ± 0.08	3.54 ± 0.78
18:3n-3	4.60 ± 0.53	11.7 ± 1.5	3.10 ± 0.26	4.3 ± 1.4
18:4n-3	14.62 ± 0.32	37.1 ± 3.2	11.4 ± 1.3	15.9 ± 5.3
18:5n-3	14.73 ± 0.13	37.4 ± 3.3	18.0 ± 2.0	25.1 ± 8.2
20:2n-6	0 ± 0	0 ± 0	0.35 ± 0.61	0.43 ± 0.74
20:3n-6	0 ± 0	0 ± 0	0.34 ± 0.59	0.41 ± 0.71
20:4n-6	0 ± 0	0 ± 0	0.27 ± 0.46	0.32 ± 0.56
20:5n-3	5.80 ± 0.14	14.7 ± 1.5	1.45 ± 0.19	1.98 ± 0.34
22:5n-3	0.14 ± 0.03	0.37 ± 0.09	0.12 ± 0.21	0.15 ± 0.26
22:6n-3	17.27 ± 0.90	43.8 ± 4.3	16.2 ± 1.8	22.0 ± 2.9
Subtotal	58.56 ± 0.85	149 ± 12	54.0 ± 1.0	74 ± 17
Total	97.27 ± 0.75	247 ± 22	96.66 ± 0.86	130 ± 34

biochemical composition is expected in two dinoflagellates coexisting in the same environment. To further complicate the identification of a biomarker for the toxic dinoflagellate, cultures of different species of *Alexandrium* grown from cysts collected in Pilley's Tickle exhibited fatty acid compositions very different than those of *A. fundyense* (Table 6.3). In fact, the *A. fundyense* culture was more similar in fatty acid composition to the unrelated *S. trochoidea* than to the two other *Alexandrium* species. In Chapter 5, the closely related diatom species, *Pseudo-nitzschia pungens* and *Pseudo-nitzschia multiseries*, were found to have a very similar fatty acid compostion so the variability encountered in these *Alexandrium* species cultured under the same conditions was unexpected. The fatty acid compostions of the two diatoms was too similar to identify a biomarker to distinguish between the two. With *Alexandrium*, it may, in fact, be very difficult to even assign a general fatty acid biomarker for that genus. It seems that the best practice would be to continue to apply biomarkers to general classes of organisms as in Chapter 4 and most of this chapter, rather than attempt to apply fatty acid biomarkers to specific organisms.

6.3.6 Biogeochemical Cycling

A clear transition was evident on going from net-tow to sediment trap to sediment samples. A decrease in PUFA occurred as more degraded material was encountered, with an increase in bacterial markers (Figure 6.6). Saturated fatty acids also increased from approximately 30% in the net-tows (Appendices II and III) to approximately 48% in the sediments (Appendix V). Most of the other fatty acids and fatty acid biomarkers followed this

Table 6.3 Proportions of major fatty acids in species of *Alexandrium*. NRC represents *A. fundyense* grown at the National Research Council laboratory in Halifax, NS. Species 5828 and 5849 were cultured at the Ocean Sciences Centre from cysts isolated from Barred Island Cove sediments (mean ± standard deviation, n=3).

	NRC	5828	5849
Fatty Acid			
14:0	10.82 ± 0.46	3.69 ± 0.48	7.63 ± 0.88
16:0	22.32 ± 0.20	22.90 ± 0.68	21.2 ± 1.4
16:1n-7	0.71 ± 0.16	42.75 ± 2.9	2.07 ± 0.35
18:1n-9	2.25 ± 0.39	1.69 ± 0.18	12.5 ± 1.6
18:3n-3	4.60 ± 0.53	1.29 ± 0.23	1.37 ± 0.06
18:4n-3	14.62 ± 0.32	1.05 ± 0.22	1.86 ± 0.38
18:5n-3	14.73 ± 0.13	2.63 ± 0.61	9.40 ± 0.68
20:5n-3	5.80 ± 0.14	3.31 ± 0.24	2.43 ± 0.61
22:6n-3	17.27 ± 0.90	1.77 ± 0.36	6.23 ± 0.77

trend by exhibiting intermediate values in the sediment traps. One of the best examples is observed in the long-chain saturated fatty acids in Barred Island Cove. These fatty acids are virtually absent in net-tow samples but comprise approximately 23% of total fatty acids in sediments (Figure 6.6c). In sediment traps, they have an average value of 1%, which is significant given their complete absence in the net-tows. This again indicates the importance of resuspension of bottom sediments in this area. This value of 1% in Barred Island Cove sediment traps may seem low but this value is larger than any encountered in any sample type in Trinity Bay.

This examination of sources and fates of organic material was similar to the one conducted in Trinity Bay (Chapter 4). A comparison of fatty acid compositions in Barred Island Cove and Trinity Bay illustrates both similarities and differences in the two environments. For example, net-tow samples in both areas contained high PUFA levels. In both Barred Island Cove and Trinity Bay, average PUFA proportions approached 50% of total fatty acids (Figure 6.6a), indicating the importance of phytoplankton sources, particularly diatoms. PUFA levels are thought to vary with temperature (Mortenson *et al*, 1988; Thompson *et al*, 1992; Renaud *et al*, 1995) and the high levels encountered in Trinity Bay may be attributed, in part, to the low water temperature (near 0 °C throughout the sampling periods) found in that area. However, in Barred Island Cove, temperatures were much warmer, fluctuating between 7 and 11 °C throughout the sampling period, suggesting that PUFA levels in phytoplankton may not, in fact, be directly related to surrounding water temperature. Other factors, such as light and nutrient availability, are known to affect PUFA

content (Harrison *et al*, 1990; Sukenik and Wahnon, 1991; Reitan *et al*, 1994; Chu *et al*, 1996).

In contrast to the PUFA proportions, levels of bacterial and terrestrial markers in net-tow and sediment trap samples in the two areas did vary considerably (Figure 6.6b). Collection of resuspended material in net-tow samples in Barred Island Cove produced significantly larger ($P<0.001$) bacterial signals than those encountered in Trinity Bay net-tows where resuspension was not an issue. Significantly higher levels ($P<0.05$) of the terrestrial markers, 18:2n-6 and 18:3n-3, were also found in net-tows and sediment traps in Barred Island Cove compared to Trinity Bay, reflecting the importance of terrestrial material as a source. Salinities in Barred Island Cove ranged from 28.5 - 31.0 practical salinity units (psu) throughout the 1995 sampling period and were lower than those found in Trinity Bay (31.6 - 32.3 psu), suggesting that fresh water runoff was delivering a portion of this terrestrial plant material into the cove. In addition, because of the proximity of the sampling site to the shore, Barred Island Cove may be particularly susceptible to contributions from airborne terrestrial material, such as pollen.

The quality of the fatty acids recovered from the sediments from the two areas was also quite different. Both compositions were dominated by saturated and monounsaturated fatty acids but in Barred Island Cove large amounts of 20:0, 22:0 and 24:0 were present as compared to monounsaturated 20:1, 22:1 and 24:1 in Trinity Bay. Long-chain monounsaturated fatty acids, particularly 20:1 and 22:1, have been associated with herbivorous zooplankton (Ratnayake and Ackman, 1979; Graeve *et al*, 1994; Albers *et al*,

1996) and their presence at proportions >6% in the sediments in Trinity Bay may indicate a substantial zooplankton source, likely through transport by fecal pellets (Libes, 1992). In Barred Island Cove sediments, the lack of long-chain monounsaturates (<1%) and the presence of 20:0, 22:0 and 24:0 (Appendix V), indicates the importance of terrestrial sources over zooplankton sources in this environment. It should be noted, however, that the terrestrial markers proposed here, 18:2n-6 and 18:3n-3, were present in much smaller proportions in Barred Island Cove than in Trinity Bay, possibly because of differences in specific terrestrial sources.

Barred Island Cove sediments also contain roughly twice as much PUFA as Trinity Bay sediments (Figure 6.6a), as well as substantial amounts of 20:5n-3 and 22:6n-3. In Trinity Bay, 20:5n-3 was present in proportions less than 1% of total fatty acids and 22:6n-3 was absent. It is suggested that reworking of these labile fatty acids during settling through depths of several hundred meters and within the sediments after deposition resulted in their low concentrations. Fluxes at this shallow site in Barred Island Cove are similar to those in Trinity Bay, with lipid fluxes ranging from 16 to 43 mg lipid m^{-2} d^{-1} in 1996 so lipid material would be susceptible to degradation within the water column for a much shorter period of time, allowing more of the large concentration of PUFA to reach the sediments intact. The presence of a blue mussel aquaculture site in this area may also have contributed to the PUFA levels. These organisms contain substantial amounts of PUFA and any bivalve exudates or decomposing bivalve tissues present in or above the sediments may be contributing to these high levels of PUFA encountered in the sediments in this small cove. In Trinity Bay, benthic

macrofauna may also be a source of PUFA (Parrish *et al*, 1996b) but their relative contribution would be expected to be much smaller as compared to Barred Island Cove which contained a bivalve aquaculture site.

6.3.7 Suitability of Fatty Acid Composition of Plankton for Bivalve Growth

The high levels of PUFA found in the net-tow samples (Appendices II and III) suggested that the plankton in Pilley's Tickle may have been particularly appropriate for growth of bivalves which require large amounts of PUFA. A survey of the recent literature (Table 6.4) suggested several optimal levels of fatty acid parameters for different bivalve growth stages. For egg and larval development, a value of 0.5 for the ratio 22:6n-3/20:5n-3 was best, while a higher value between 1 and 2 was more appropriate for adult growth. The ratio of n-3/n-6 PUFA was best between 5 and 15, regardless of growth stage. To investigate the suitability of this phytoplankton for adult bivalve growth in light of these optimal values, the fatty acid composition of blue mussels from this site was determined (Table 6.5).

It was originally thought that it would be possible to identify a fatty acid marker in the bivalve that would indicate the presence of toxic *A. fundyense* in its diet. However, the fatty acid composition of the blue mussel examined here was very similar to that reported in the literature (Joseph, 1982; Joseph, 1989; Pranal *et al*, 1997) and in Section 3.3.1.2 with 20:5n-3 and 22:6n-3 comprising approximately 30% of total fatty acids (Table 6.5). A suitable biomarker for *A. fundyense* was not apparent. Interestingly, 18:5n-3 was not identified in the bivalve, although dinoflagellates containing this fatty acid presumably comprised a significant

Table 6.4 Literature values of 22:6n-3/20:5n-3 in bivalve eggs, larvae and adults.

Eggs and Larvae	Adults	Reference
0.35	1.1	Napolitano *et al* (1992)
0.26 - 0.65	-----	Pazos *et al* (1997)
0.57	-----	Whyte *et al* (1991)
0.55	-----	Marty *et al* (1992)
-----	1.0 - 1.5	Joseph (1982)
-----	2	Parrish *et al* (1999)
-----	1	Pranal *et al* (1997)

Table 6.5 Fatty acid composition (% total fatty acids and DMA) of *Mytelis edulis* collected in Barred Island Cove in 1996 (mean ± standard deviation, n = 3).

Fatty Acid		Fatty Acid	
Branched		Polyunsaturated	
i-15:0	0.08 ± 0.07	16:2	0.53 ± 0.02
ai-15:0	0.07 ± 0.06	18:2n-6	1.64 ± 0.20
i-16:0	0.20 ± 0.03	18:2n-4	0.19 ± 0.05
i-17:0	0.52 ± 0.04	18:3n-6	0.10 ± 0.09
ai-17:0	0.65 ± 0.14	18:3n-4	0.18 ± 0.02
i-18:0	0.14 ± 0.01	18:3n-3	1.61 ± 0.13
Subtotal	1.67 ± 0.24	18:4n-3	2.66 ± 0.29
		20:2Δ5,11	2.35 ± 0.77
Saturated		20:2n-6	0.51 ± 0.06
14:0	4.05 ± 0.68	20:3 NMI	0.50 ± 0.05
15:0	0.73 ± 0.08	20:4n-6	2.56 ± 0.49
16:0	13.4 ± 1.8	20:3n-3	0.08 ± 0.07
17:0	0.43 ± 0.11	20:4n-3	0.30 ± 0.08
18:0	2.36 ± 0.18	20:5n-3	16.0 ± 1.8
Subtotal	21.0 ± 1.5	22:2Δ7,13	0.43 ± 0.12
		22:2Δ7,15	3.08 ± 0.46
Monounsaturated		22:3	1.29 ± 0.25
16:1n-7	8.8 ± 1.4	22:4n-6	0.21 ± 0.06
16:1n-5	0.21 ± 0.02	22:5n-6	0.33 ± 0.03
18:1n-9	1.92 ± 0.14	22:5n-3	0.96 ± 0.06
18:1n-7	2.64 ± 0.33	22:6n-3	15.0 ± 1.9
18:1n-5	0.17 ± 0.04	Subtotal	50.6 ± 1.7
20:1n-11	1.11 ± 0.15		
20:1n-9	2.71 ± 0.29	Dimethyl Acetals	
20:1n-7	0.92 ± 0.44	16:0 DMA	0.43 ± 0.08
Subtotal	18.5 ± 1.5	i-18:0 DMA	0.38 ± 0.14
		18:0 DMA	4.51 ± 0.89
		20:0 DMA	0.97 ± 0.21
		Subtotal	6.3 ± 1.3
		Total	97.97 ± 0.34

portion of the bivalve's diet. The unusual non-methylene interrupted dienoic and trienoic fatty acids previously reported (Joseph, 1982; Zhukova, 1986; Zhukova, 1991) were also identified and double bond positions were determined in the dienes. Several DMA, including 16:0, *i*-18:0, 18:0 and 18:1 were identified using GC-MS. Fragmentation patterns of DMA were characterized by large signals at *m/z* 75, representing the dimethyl acetal fragment ($HC(OMe)_2^+$), and M-31, representing loss of OMe. Both *i*-18:0 and 18:1 were present in small amounts, and 18:1 DMA comprised less than 0.10% of total fatty acids so was not included in Table 6.5. This is the first report of *i*-18:0 DMA and 18:1 DMA in a bivalve. DMA are generated when vinyl ether-linked lipids are treated with acid in the presence of methanol, and bivalves are known to contain plasmalogens with that type of ether linkage (Joseph, 1982). These plasmalogens are thought to play a role as membrane components and some ether-linked lipids serve as protective storage areas for PUFA. The ether group slows the rate of hydrolysis of the acyl chain in the *sn*-2 position so that PUFA in that location may be better retained in the phospholipid (Snyder, 1987).

Several important fatty acid parameters in the blue mussel were compared to those of the average plankton composition in 1995 and 1996 (Figure 6.8a). Average values of 22:6n-3/20:5n-3 and n-3/n-6 PUFA ratios over the two year period were near the optimal ranges for adult bivalve growth with values in 1995 of 1.0 ± 0.9 and 12.7 ± 5.2, and in 1996 of 0.8 ± 0.3 and 10.8 ± 1.6, respectively. Furthermore, the similarities in profiles of fatty acid parameters in the plankton and mussel samples were clear. PUFA levels and the 22:6n-3/ 20:5n-3 ratios were comparable in both sample types, while there was less similarity in

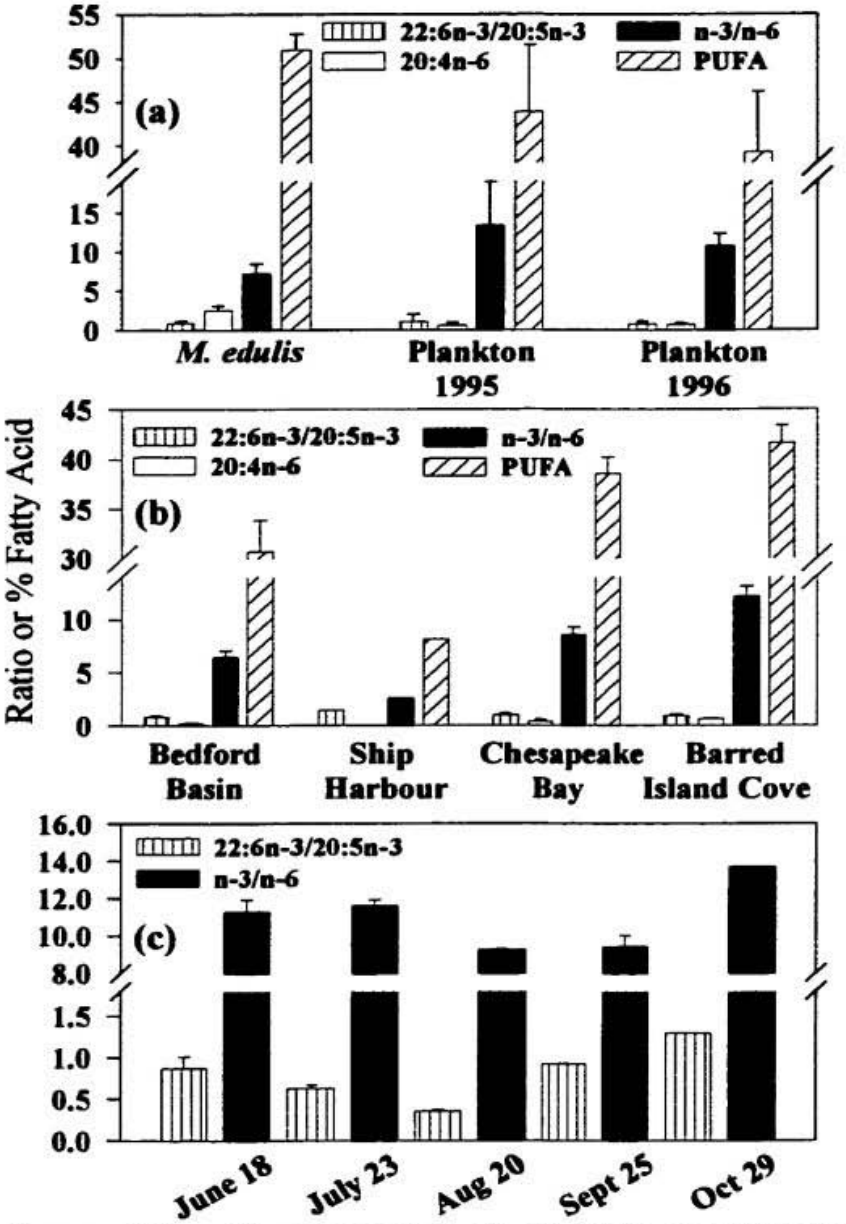


Figure 6.8 Average fatty acid parameters in (a) *M. edulis* and phytoplankton samples collected in Notre Dame Bay in 1995 and 1996 and (b) phytoplankton samples in other areas. In (c) fluctuations of fatty acids parameters in net-tow samples in 1996 are displayed (mean ± standard deviation, n = 1-10).

20:4n-6 and n-3/n-6 PUFA. Proportions of 20:4n-6 were 5-fold greater in the mussel than in the plankton, suggesting that the mussel can selectively retain this fatty acid. This higher level of 20:4n-6 in the bivalve also contributed to the reduced n-3/n-6 PUFA levels because 20:4n-6 was the major n-6 fatty acid.

Clearly, the plankton were providing n-3 fatty acids in proportions very similar to the bivalve's requirements but, while the fatty acid composition of the bivalves was typical of blue mussels found elsewhere (Joseph, 1982), the fatty acid composition of the plankton was unique. The fatty acid composition of seston from other sheltered areas (Mayzaud *et al*, 1989; Parrish *et al*, 1992; Harvey and Johnston, 1995) does not exhibit such optimal values (Figure 6.8b). The fatty acid composition of seston in Chesapeake Bay, which supports a successful oyster fishery, is most similar to that of Barred Island Cove but even that site contains lower PUFA, 20:4n-6 and n-3/n-6 levels than Barred Island Cove plankton.

6.3.8 Synchronization of Fatty Acid Parameters with Bivalve Development

The timing of fluctuations of fatty acid parameters in the diet of early growth stages of bivalves in the natural environment may be critical. The optimal values outlined in Section 6.3.7, combined with the fatty acid compositions of net-tow data from Barred Island Cove in 1996 may be used to examine this timing. The 22:6n-3/20:5n-3 and n-3/n-6 PUFA ratios of plankton samples collected in 1996 are shown in Figure 6.8c. It is known that in this area spawning of the blue mussel occurs in mid-June. The sample collected on June 18 had a 22:6n-3/20:5n-3 value of approximately 0.8, which is larger than the optimum for egg

development. However, with spawning in June, the final stages of gonad and egg development must have occurred in late May and early June and it was diatom bloom material which usually generates a low 22:6n-3/20:5n-3 ratio, appropriate for egg development, on which the organism would have been feeding. Furthermore, larvae would have been present between the June 18 and July 23 sampling dates and would have been feeding on plankton (specifically diatoms) having a 22:6n-3/20:5n-3 ratio between 0.6 and 0.8. The lowest of these values was only slightly greater than the 0.5 optimum, which is adequate for larval development. After the larval stage, ideally, higher values of the 22:6n-3/20:5n-3 ratio, more appropriate for mature organisms, would have been found in the plankton. However, the lowest 22:6n-3/20:5n-3 value of any sampling date was found on August 20 when spat would have been present, due to the presence of *Skeletonema costatum*, *Chaetoceros* sp. and *Nitzschia closterium*. Although this ratio seems to be inappropriate, one could argue that larval survival is the critical factor and that the more robust spat stage would be better able to accommodate a less favourable 22:6n-3/20:5n-3 ratio. After August 20, higher 22:6n-3/20:5n-3 ratios, with values near 1, appropriate for adult bivalves, were found in the plankton samples. Although the n-3/n-6 PUFA ratio did not fluctuate greatly, it always had a value between approximately 9 and 14, which was within the optimal range for eggs, larvae and adults.

6.4 Conclusions

High levels of PUFA were found in the net-tow and sediment trap samples, indicating that a large proportion of this material had a marine phytoplankton source. The fatty acid biomarker levels in net-tow samples reflected the changing abundances of diatoms and dinoflagellates, and agreed with microscopic determinations of those organisms. Fatty acid biomarkers also indicated surprisingly high bacterial levels in the net-tow and sediment trap samples, likely a result of the collection of resuspended sediment in these samples. The terrestrial markers, 18:2n-6 and 18:3n-3, were also high compared to Trinity Bay in both net-tow and sediment trap samples, reflecting the input of fresh water runoff and terrestrial plant material in the cove. Stable carbon isotope analysis of those two fatty acids indicated that they did have a predominant terrestrial origin. Fatty acid analysis of vegetative cultures of toxic *A. fundyense* and non-toxic *S. trochoidea* did not reveal a suitable marker to distinguish between the two organisms. However, the NRC strains of both dinoflagellates did contain 18:5n-3 and 22:6n-3 in amounts greater than 30% of total, offering support for the choice of the general dinoflagellate markers employed in this study.

A very different fatty acid composition was encountered in the sediments, with saturated and monounsaturated fatty acids predominating. The terrestrial markers, 20:0, 22:0 and 24:0, and bacterial markers were present in large proportions, reflecting their importance as sources. PUFA levels were low, suggesting that most of these labile fatty acids were effectively recycled, either before reaching the sediments or after deposition.

The fatty acid composition of blue mussels collected in Barred Island Cove was very similar to healthy mussels collected elsewhere (Joseph, 1982) and a suitable marker to indicate the presence of the toxic dinoflagellate, *A. fundyense*, in the diet of the bivalve was not apparent. PUFA levels in the blue mussel were near 50% of total fatty acids with 20:5n-3 and 22:6n-3 comprising 16% and 15% of total, respectively. Trace amounts of the unusual *i*-18:0 DMA and 18:1 DMA were also identified in these bivalves. The average fatty acid composition of net-tow samples containing fresh plankton material agreed well with that of the adult blue mussels, suggesting that the plankton were providing fatty acids in proportions very similar to the bivalve's requirements. Fatty acid parameters in the plankton also fluctuated with a timing that was consistent with the changing needs of bivalves at different growth stages. Ironically, in Barred Island Cove, it seems that the conditions that promote the formation of cysts of toxic *A. fundyense* also encourage growth of phytoplankton with fatty acid compositions suitable for bivalve growth. These fatty acid compositions of plankton harvested in Barred Island Cove are likely typical of plankton throughout the Tickle, suggesting that other coves within Pilley's Tickle in Notre Dame Bay where toxic algae are not present may also contain plankton that are particularly appropriate for bivalve aquaculture in terms of fatty acid nutrition.

Chapter 7 - CONCLUSIONS

Information gained from fatty acid analyses of marine samples is useful in a variety of fields. However, before fatty acid data can be applied to environmental studies, there must be some guarantee that the results are accurate and reproducible. For that purpose, the methods and techniques typically employed to generate fatty acid data were critically evaluated. The choice of appropriate extraction technique depended on sample type, while any among four acid catalyzed methods could be used for FAME synthesis. Column chromatography was used to separate extracts into neutral, AMPL and PL fractions and to remove hydrocarbon contamination. Surprisingly, losses of PL occurred on the column, which illustrates the importance of independently confirming results generated by a particular technique. Treatment of samples with high lipase activities, such as diatoms, with boiling water prior to extraction was found to be very effective in deactivating those enzymes. Without boiling water treatment, there were high levels of free fatty acids, a breakdown product. Finally, the utility of picolinyl derivatives of fatty acids for structure elucidation was evaluated and a new method for their formation was developed.

Fatty acid data are not consistently reported as concentrations perhaps because there is a hesitancy in many laboratories to add an internal standard that may coelute with a naturally occurring fatty acid. Lipid class data, generated by TLC-FID, however, are usually reported as concentrations. A series of calculations, designed to incorporate fatty acid proportions with acyl lipid class concentrations to arrive at fatty acid concentrations, was

developed. These estimates increased in accuracy as knowledge of the non-acyl structures of acyl lipids increased, and were in good agreement with actual fatty acid concentrations, determined using internal standards, in algae and animal samples. Applications of this calculation to sediments was not as successful, but it did prompt an in-depth examination of the AMPL and PL fractions of a sediment sample. Interestingly, the bulk of the PL fraction was found to consist of a non-acyl lipid of undetermined structure. Peaks that may be the monoacylglycerol derivatives of mono- and digalactosyl diacylglycerols were also found in the AMPL fraction. Structure determination of these peaks would be an exciting future project, as there is virtually no information in the literature concerning glycolipids and PL in sediments.

When fatty acid compositions of marine samples are known with confidence, it becomes possible to apply them as biomarkers of various sources. In this thesis, several general biomarkers for diatoms, dinoflagellates, bacteria and terrestrial material were applied to a variety of samples. In both Chapters 4 and 6, these markers agreed well, in most cases, with phytoplankton compositions determined floristically. The differences in environment in Barred Island Cove and Trinity Bay were also evident in the different distributions of bacterial and terrestrial markers in net-tows, sediment traps and sediments. It should be stressed that these fatty acid biomarkers were successful as indicators of source only when applied generally to classes of organisms.

The use of fatty acid biomarkers as indicators of specific species was also investigated in Chapter 5 and 6. The closely related diatoms *Pseudo-nitzschia multiseries* and *Pseudo-*

nitzschia pungens had very similar fatty acid compositions and a suitable marker to distinguish between the two was not identified. However, the fatty acid compositions of three species of the dinoflagellate *Alexandrium* were quite different. In fact, the fatty acid compositions of *A. fundyense* was more similar to the unrelated dinoflagellate *Scrippsiella trochoidea* than to any other *Alexandrium* species. This suggests that, with both the diatoms and dinoflagellates, it is probably not reasonable to expect to identify a biomarker suitable for use in the field. Nevertheless, a marker to differentiate these similar-appearing organisms from one another in the laboratory would be useful. In any case, this suggests that the use of fatty acid biomarkers as indicators of specific organisms must be evaluated on a case-by-case basis.

Finally, the suitability of phytoplankton in terms of fatty acid composition in the diet of bivalves was examined. Often PUFA levels are the only parameter considered but, here, appropriate ratios of n-3/n-6 PUFA and 22:6n-3/20:5n-3 were determined from the literature for egg, larvae and adult bivalves. Fluctuations in those parameters over time in phytoplankton in Barred Island Cove seemed to correspond with the changing needs of the bivalves when developing from eggs to larvae to more mature stages. The average values of those parameters were also very similar to those encountered in typical bivalves harvested in Barred Island Cove and elsewhere. Ironically, these optimal fatty acid compositions are found in the phytoplankton at a site that is permanently closed to aquaculture because of the presence of toxic dinoflagellate cysts. In the future, before establishing aquaculture sites, it may be useful to consider the fatty acid compositions of naturally occurring plankton populations.

To summarize, this thesis has demonstrated the usefulness of accurate fatty acid analysis of marine samples. Temporal and spatial fluctuations in fatty acid compositions of marine samples can give insight into biogeochemical processes. Fatty acid biomarkers may also be used quite successfully if one's expectations are realistic; the use of specific fatty acid biomarkers for single species is probably not feasible. Lastly, fatty acid compositions may be used in nutritional studies in aquaculture to aid in the evaluation of the ability of a particular diet to meet the bivalve's fatty acid requirements.

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Appendix I. Results of calculations to produce FAME concentrations from FAME proportions and lipid class concentrations in *Nannochloropsis* sp.

Fatty Acid	Molar Mass	% Weight	Moles (x1000)	Mole %
14:0	242.4	5.61	23.14	6.94
14:1n-5	240.384	0.31	1.29	0.39
i-15:0	256.427	1.11	4.33	1.30
15:0	256.427	0.30	1.17	0.35
ai-16:0	270.454	1.40	5.18	1.55
16:0	270.454	16.20	59.90	17.95
16:1n-9	268.438	3.43	12.78	3.83
16:1n-7	268.438	19.35	72.08	21.61
i-17:0	284.481	0.19	0.67	0.20
ai-17:0	284.481	0.38	1.34	0.40
16:2n-4	266.422	0.29	1.09	0.33
17:0	284.481	0.22	0.77	0.23
16:3n-4	264.406	0.76	2.87	0.86
16:4n-1	262.391	0.24	0.91	0.27
18:0	298.508	0.33	1.11	0.33
18:1n-9	296.492	3.30	11.13	3.34
18:1n-7	296.492	0.50	1.69	0.51
18:2n-6	294.476	4.28	14.53	4.36
18:3n-6	292.461	0.43	1.47	0.44
18:3n-3	292.461	0.19	0.65	0.19
20:4n-6	318.498	4.14	13.00	3.90
20:5n-3	316.483	32.45	102.53	30.73
23:0				
Total		95.41	333.63	100
Avg Chain Length		17.41		
Avg Double Bonds		2.14		
Average Molecular Weight		285.97		

Amount (µg in extract)

Lipid Class (by TLC/FID)		FAME derived from acyl lipids	
Hydrocarbons	64	Steryl/Wax Esters	0
Steryl/Wax Esters	0	Methyl Esters	1
Methyl Esters	1	Ketones	0
Ketones	7	Triacylglycerols	134
Triacylglycerols	134	Free Fatty Acids	16
Free Fatty Acids	15	Diacylglycerols	6
Alcohols	2	AMPL	76
Sterols	39	Phospholipids	250
Diacylglycerols	6		
AMPL	370	Total Lipids	483
Polar Lipids	335		
Total	973		

Appendix II. Fatty acid composition (% total fatty acids) of net-tow samples collected in Barred Island Cove in 1995 (mean ± standard deviation, n =1-5).

Fatty Acid	Aug 29	Aug 31	Oct 4	Oct 24	Nov 14
Branched					
i-15:0	0.33 ± 0.08	0.36	0.78 ± 0.13	0.35 ± 0.14	0.58 ± 0.19
ai-15:0	0.45 ± 0.08	0.56	0.56 ± 0.10	0.33 ± 0.13	0.04 ± 0.07
i-16:0	0 ± 0	0.17	0.70 ± 0.30	0.21 ± 0.04	0.35 ± 0.04
ai-16:0	0.27 ± 0.10	0.39	0.8 ± 1.4	0.34 ± 0.10	0.81 ± 0.03
i-17:0	0.91 ± 0.01	0.93	0.68 ± 0.10	1.19 ± 0.08	2.61 ± 0.07
ai-17:0	0.88 ± 0.29	0.64	0.31 ± 0.06	0 ± 0	0.77 ± 0.02
Subtotal	2.83 ± 0.21	3.05	3.9 ± 1.3	2.41 ± 0.42	5.15 ± 0.17
Saturated					
14:0	19.40 ± 0.06	13.95	7.84 ± 0.45	6.0 ± 3.6	12.22 ± 0.38
15:0	0.78 ± 0.04	1.26	1.09 ± 0.10	0.41 ± 0.10	0.42 ± 0.01
16:0	12.02 ± 0.62	14.09	16.9 ± 1.7	16.52 ± 0.81	8.90 ± 0.31
17:0	0.14 ± 0.02	0.28	0.44 ± 0.06	0.29 ± 0.07	0 ± 0
18:0	1.17 ± 0.52	1.56	2.13 ± 0.17	2.29 ± 0.46	0.69 ± 0.02
20:0	0 ± 0	0.14	0.37 ± 0.25	0.20 ± 0.05	0.04 ± 0.06
22:0	0 ± 0	0	0.17 ± 0.04	0 ± 0	0 ± 0
Subtotal	33.5 ± 1.1	31.28	28.9 ± 2.0	25.7 ± 4.0	22.27 ± 0.78
Monounsaturated					
16:1n-9	0 ± 0	0.97	0.66 ± 0.26	0.50 ± 0.13	1.86 ± 0.21
16:1n-7	26.78 ± 0.89	24.10	9.7 ± 1.8	3.38 ± 0.36	17.32 ± 0.43
16:1n-5	0.29 ± 0.40	0.48	0.70 ± 0.10	0.52 ± 0.05	0.38 ± 0.01
18:1n-9	1.13 ± 0.04	1.44	6.1 ± 1.1	8.6 ± 1.4	1.44 ± 0.16
18:1n-7	0.66 ± 0.09	1.03	2.01 ± 0.34	0.73 ± 0.25	1.08 ± 0.14
18:1n-5	0 ± 0	0.24	0.28 ± 0.27	0.22 ± 0.06	0.17 ± 0.01
20:1n-11	0 ± 0	0.21	0.20 ± 0.05	0.16 ± 0.03	0 ± 0
20:1n-7	0 ± 0	0.16	0.30 ± 0.05	0.31 ± 0.03	0.09 ± 0.08
22:1n-11	0.30 ± 0.12	0	0.30 ± 0.53	0 ± 0	0 ± 0
22:1n-9	0 ± 0	0	0.07 ± 0.13	0.15 ± 0.03	0.08 ± 0.07
24:1	0 ± 0	0	0.06 ± 0.10	0 ± 0	0 ± 0
Subtotal	29.20 ± 0.42	28.63	20.4 ± 2.8	14.55 ± 0.92	22.41 ± 0.41
Polyunsaturated					
16:2n-4	2.03 ± 0.14	1.83	0.89 ± 0.17	0.56 ± 0.06	3.69 ± 0.07
16:3n-4	2.00 ± 0.05	1.59	1.37 ± 0.28	0.54 ± 0.07	3.68 ± 0.06
16:4n-3	0.20 ± 0.03	0.42	1.2 ± 1.2	0.71 ± 0.12	0 ± 0
16:4n-1	3.45 ± 0.21	2.54	2.0 ± 1.8	0.92 ± 0.10	11.30 ± 0.14
18:2n-6	1.23 ± 0.03	1.18	2.24 ± 0.16	2.24 ± 0.15	0.69 ± 0.03
18:2n-4	0 ± 0	0	0.17 ± 0.05	0 ± 0	0 ± 0
18:3n-6	0.45 ± 0.01	0.41	0.25 ± 0.08	0.11 ± 0.10	0.31 ± 0.01
18:3n-3	0.46 ± 0.01	0.64	1.31 ± 0.06	1.36 ± 0.05	0.21 ± 0.02
18:4n-3	2.23 ± 0.11	2.33	2.91 ± 0.19	4.12 ± 0.41	2.49 ± 0.03
18:5n-3	0.30 ± 0.03	0.76	3.47 ± 0.52	9.72 ± 0.45	0.52 ± 0.02
20:2n-6	0.08 ± 0.11	0.18	0.67 ± 0.35	0.31 ± 0.05	0 ± 0
20:3n-6	0.19 ± 0.01	0.15	0 ± 0	0 ± 0	0 ± 0
20:3n-3	0 ± 0	0	0.08 ± 0.13	0.21 ± 0.03	0 ± 0
20:4n-6	0.44 ± 0.06	0.57	0.70 ± 0.12	0.96 ± 0.14	0.26 ± 0.01
20:4n-3	0.54 ± 0.03	0.70	0.73 ± 0.05	0.94 ± 0.07	0.60 ± 0.06
20:5n-3	14.6 ± 1.7	14.04	9.0 ± 1.2	9.54 ± 0.52	19.0 ± 1.7
22:5n-3	0.18 ± 0.01	0.27	0.43 ± 0.14	0.52 ± 0.06	0.16 ± 0.01
22:6n-3	3.58 ± 0.42	6.12	10.3 ± 3.8	21.3 ± 2.3	2.83 ± 0.09
Subtotal	32.0 ± 2.5	33.73	37.8 ± 4.5	54.0 ± 3.4	45.7 ± 1.4
Total	97.5 ± 1.2	96.69	90.9 ± 1.2	96.66 ± 0.74	95.55 ± 0.31

Appendix III. Fatty acid composition (% total fatty acids) of net-tow samples collected in Barred Island Cove in 1996 (mean ± standard deviation, n =1-2).

Fatty Acid	June 18	July 23	Aug 20	Sept 25	Oct 29
Branched					
i-15:0	0.97 ± 0.24	0.39 ± 0.01	0.41 ± 0.00	0.63 ± 0.08	0.52
ai-15:0	0.86 ± 0.52	0.45 ± 0.05	0.64 ± 0.03	0.64 ± 0.07	0.43
i-16:0	0.58 ± 0.42	0.13 ± 0.01	0.12 ± 0.01	0.32 ± 0.01	0.15
ai-16:0	0 ± 0	0.14 ± 0.19	0.41 ± 0.00	0.09 ± 0.12	0.13
i-17:0	1.31 ± 0.17	0.97 ± 0.04	0.98 ± 0.01	0.98 ± 0.11	0.82
ai-17:0	0.19 ± 0.26	0.57 ± 0.08	0.34 ± 0.04	0.26 ± 0.04	0.00
Subtotal	3.9 ± 1.1	2.64 ± 0.01	2.89 ± 0.01	2.90 ± 0.42	2.05
Saturated					
14:0	8.8 ± 1.9	16.95 ± 0.50	16.19 ± 0.25	6.70 ± 0.58	4.94
15:0	1.5 ± 1.0	0.84 ± 0.01	1.03 ± 0.01	1.13 ± 0.11	1.09
16:0	22.5 ± 2.2	13.64 ± 0.13	13.31 ± 0.13	20.5 ± 1.2	17.48
17:0	0.44 ± 0.62	0.33 ± 0.08	0.31 ± 0.01	0.95 ± 0.10	0.63
18:0	2.70 ± 0.38	1.04 ± 0.05	1.54 ± 0.08	3.33 ± 0.45	1.97
20:0	0.21 ± 0.30	0.08 ± 0.11	0 ± 0	0 ± 0	0.00
Subtotal	36.2 ± 3.8	32.87 ± 0.53	32.37 ± 0.47	32.6 ± 2.4	26.11
Monounsaturated					
15:1	0 ± 0	0 ± 0	0.20 ± 0.01	0 ± 0	0.00
16:1n-7	8.45 ± 0.31	24.14 ± 0.42	22.65 ± 0.23	8.50 ± 0.00	5.02
16:1n-5	1.14 ± 0.42	0.73 ± 0.03	0.51 ± 0.01	0.77 ± 0.13	0.52
18:1n-9	2.64 ± 0.49	1.39 ± 0.10	1.63 ± 0.01	7.4 ± 2.8	7.16
18:1n-7	2.40 ± 0.28	1.29 ± 0.23	1.29 ± 0.01	3.63 ± 0.22	2.13
18:1n-5	0.41 ± 0.00	0.06 ± 0.08	0.05 ± 0.06	0.53 ± 0.01	0.28
20:1n-11	0 ± 0	0 ± 0	0 ± 0	0.09 ± 0.13	0.00
20:1n-9	0.70 ± 0.98	0 ± 0	0.12 ± 0.02	0.29 ± 0.01	0.56
20:1n-7	0.94 ± 0.63	0.15 ± 0.02	0.07 ± 0.10	0.29 ± 0.03	0.18
Subtotal	16.7 ± 1.0	27.75 ± 0.06	26.50 ± 0.07	21.5 ± 2.5	15.85
Polyunsaturated					
16:2n-4	0.45 ± 0.09	2.01 ± 0.06	2.05 ± 0.03	0.77 ± 0.02	0.51
16:3n-4	0.66 ± 0.13	1.04 ± 0.13	1.21 ± 0.03	0.58 ± 0.05	0.45
16:4n-3	0.95 ± 0.01	0.38 ± 0.08	0.32 ± 0.02	0.79 ± 0.05	1.00
16:4n-1	0.72 ± 0.07	4.00 ± 0.23	3.21 ± 0.01	0.64 ± 0.03	0.66
18:2n-6	1.62 ± 0.37	1.00 ± 0.10	1.25 ± 0.01	2.32 ± 0.12	2.49
18:3n-6	0 ± 0	0.44 ± 0.09	0.36 ± 0.01	0.09 ± 0.12	0.00
18:3n-3	1.16 ± 0.16	0.61 ± 0.01	0.53 ± 0.00	1.84 ± 0.21	2.06
18:4n-3	3.82 ± 0.52	2.91 ± 0.04	2.01 ± 0.08	3.84 ± 0.48	6.03
18:5n-3	1.77 ± 0.21	1.26 ± 0.10	0.91 ± 0.08	0.95 ± 0.04	1.44
20:2n-6	0.19 ± 0.26	0 ± 0	0.11 ± 0.01	0.34 ± 0.06	0.46
20:3n-6	0 ± 0	0.16 ± 0.03	0.18 ± 0.01	0 ± 0	0.00
20:4n-6	0.55 ± 0.00	0.60 ± 0.09	0.91 ± 0.00	0.86 ± 0.01	0.54
20:3n-3	0 ± 0	0 ± 0	0 ± 0	0.22 ± 0.08	0.27
20:4n-3	0.59 ± 0.13	0.56 ± 0.02	0.56 ± 0.01	1.02 ± 0.23	2.35
20:5n-3	12.3 ± 1.9	11.8 ± 1.2	16.35 ± 0.10	13.60 ± 0.21	15.30
22:3	0.42 ± 0.07	0 ± 0	0 ± 0	0.29 ± 0.01	0.37
22:4n-3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.12
22:5n-6	0.52 ± 0.11	0 ± 0	0 ± 0	0.16 ± 0.00	0.11
22:5n-3	0.67 ± 0.27	0.41 ± 0.06	0.28 ± 0.01	0.48 ± 0.01	0.74
22:6n-3	10.9 ± 3.2	7.52 ± 0.37	5.80 ± 0.30	12.53 ± 0.08	19.93
Subtotal	37.2 ± 7.5	34.7 ± 1.2	36.11 ± 0.47	41.28 ± 0.69	54.83
Total	94.0 ± 3.5	97.93 ± 0.58	97.86 ± 0.07	98.26 ± 0.35	98.84

Appendix IV. Fatty acid composition (% total fatty acids) of sediment trap samples collected in Barred Island Cove in 1995 and1996 (mean ± standard deviation, n =3-4).

Fatty Acid	Aug 95	Nov 95	Oct 96
Branched			
i-15:0	0.41 ± 0.05	1.12 ± 0.03	1.36 ± 0.50
ai-15:0	0.58 ± 0.28	0.67 ± 0.15	0.80 ± 0.29
i-16:0	0.28 ± 0.04	0.51 ± 0.02	0.58 ± 0.23
ai-16:0	0.42 ± 0.04	0.78 ± 0.26	0.70 ± 0.31
i-17:0	1.27 ± 0.40	1.28 ± 0.08	0.87 ± 0.17
ai-17:0	0.48 ± 0.46	0.72 ± 0.11	0.66 ± 0.32
Subtotal	3.45 ± 0.55	5.06 ± 0.13	5.0 ± 1.5
Saturated			
14:0	9.9 ± 1.1	8.00 ± 0.31	5.97 ± 0.90
15:0	1.03 ± 0.25	1.16 ± 0.07	1.78 ± 0.34
16:0	15.02 ± 0.59	14.0 ± 1.4	19.0 ± 7.3
17:0	0.40 ± 0.04	0.39 ± 0.08	0.61 ± 0.29
18:0	2.35 ± 0.29	2.77 ± 0.62	4.7 ± 4.7
20:0	0.20 ± 0.01	0.38 ± 0.12	0.39 ± 0.09
22:0	0.23 ± 0.02	0.27 ± 0.05	0.30 ± 0.12
24:0	0.50 ± 0.13	0.41 ± 0.19	0.38 ± 0.19
Subtotal	29.61 ± 0.79	27.3 ± 1.3	33 ± 12
Monounsaturated			
15:1	0.28 ± 0.09	0.35 ± 0.21	0.34 ± 0.13
16:1n-9	0.16 ± 0.27	1.60 ± 0.48	1.22 ± 0.69
16:1n-7	17.58 ± 0.27	16.38 ± 0.46	12.7 ± 4.1
16:1n-5	0.10 ± 0.17	0.51 ± 0.01	0.61 ± 0.22
18:1n-9	3.2 ± 1.1	3.00 ± 0.60	5.1 ± 3.6
18:1n-7	1.97 ± 0.29	3.7 ± 2.1	4.77 ± 0.50
18:1n-5	0 ± 0	0.14 ± 0.20	0.17 ± 0.19
20:1n-9	0.17 ± 0.15	0.25 ± 0.12	0.35 ± 0.14
20:1n-7	0.23 ± 0.03	0.29 ± 0.01	0.53 ± 0.10
22:1n-11	0 ± 0	0.26 ± 0.36	0 ± 0
22:1n-9	0.26 ± 0.14	0.49 ± 0.50	0.18 ± 0.13
24:1	0.10 ± 0.09	0.16 ± 0.01	0.13 ± 0.16
Subtotal	24.06 ± 0.99	27.1 ± 2.7	26.1 ± 1.7
Polyunsaturated			
16:2n-4	1.40 ± 0.06	2.50 ± 0.18	1.42 ± 0.82
16:3n-4	1.43 ± 0.09	3.19 ± 0.26	3.1 ± 1.5
16:4n-3	1.37 ± 0.12	0.46 ± 0.07	0.53 ± 0.17
16:4n-1	1.36 ± 0.06	4.21 ± 0.91	1.10 ± 0.58
18:2n-6	1.64 ± 0.12	1.60 ± 0.15	1.65 ± 0.64
18:2n-4	0.04 ± 0.08	0 ± 0	0.53 ± 0.54
18:3n-6	0.37 ± 0.04	0.27 ± 0.00	0.33 ± 0.08
18:3n-4	0 ± 0	0.12 ± 0.17	0.05 ± 0.10
18:3n-3	1.76 ± 0.04	0.83 ± 0.17	1.16 ± 0.35
18:4n-3	2.80 ± 0.13	2.27 ± 0.59	2.06 ± 0.08
18:5n-3	2.33 ± 0.17	0.97 ± 0.03	1.17 ± 0.30
20:2n-6	0.38 ± 0.11	0.31 ± 0.04	0.55 ± 0.14
20:3n-6	0.12 ± 0.00	0.22 ± 0.30	0.07 ± 0.08
20:3n-3	0.46 ± 0.14	0.06 ± 0.08	0.03 ± 0.06
20:4n-6	1.10 ± 0.13	0.97 ± 0.16	1.33 ± 0.64
20:4n-3	0.52 ± 0.06	0.41 ± 0.11	0.38 ± 0.11
20:5n-3	12.62 ± 0.74	11.9 ± 1.7	9.4 ± 4.5
22:5n-6	0.38 ± 0.08	0.09 ± 0.12	0.10 ± 0.11
22:5n-3	0.66 ± 0.61	0.26 ± 0.04	0.29 ± 0.08
22:6n-3	9.54 ± 0.61	3.87 ± 0.04	5.5 ± 1.2
Subtotal	40.28 ± 0.78	34.5 ± 3.6	30.7 ± 6.3
Total	97.40 ± 0.99	93.91 ± 0.32	94.8 ± 2.7

Appendix V. Fatty acid composition (% total fatty acids) of sediments collected in Barred Island Cove in 1995 (mean ± standard deviation, n = 6 sediment samples).

Fatty Acid		
Branched		
i-15:0	2.23 ±	0.41
ai-15:0	2.79 ±	0.76
i-17:0	0.57 ±	0.49
ai-17:0	0.87 ±	0.24
Subtotal	6.5 ±	1.4
Saturated		
14:0	5.07 ±	0.55
15:0	1.60 ±	0.65
16:0	13.5 ±	2.4
17:0	0.50 ±	0.29
18:0	3.82 ±	0.76
20:0	3.3 ±	1.0
22:0	8.3 ±	1.7
24:0	11.8 ±	2.6
Subtotal	48.0 ±	5.7
Monounsaturated		
14:1	0.59 ±	0.46
15:1	1.45 ±	0.39
16:1n-9	0.24 ±	0.40
16:1n-7	9.23 ±	0.71
16:1n-5	2.9 ±	2.0
18:1n-11	0.19 ±	0.30
18:1n-9	4.4 ±	2.5
18:1n-7	4.02 ±	0.70
18:1n-5	0.26 ±	0.32
20:1n-11	0.20 ±	0.23
20:1n-9	0.15 ±	0.24
20:1n-7	0.31 ±	0.25
Subtotal	23.9 ±	2.1
Polyunsaturated		
16:2n-4	2.10 ±	0.59
16:3n-4	1.79 ±	0.57
18:2n-6	0.68 ±	0.16
18:3n-3	0.84 ±	0.45
18:4n-3	0.37 ±	0.19
20:4n-6	1.56 ±	0.56
20:3n-3	0.34 ±	0.57
20:5n-3	5.3 ±	2.4
22:6n-3	1.2 ±	1.9
Subtotal	14.1 ±	4.0
Total	92.5 ±	3.9