THE BIOGEOCHEMISTRY OF STEROLS IN TRINITY BAY, NEWFOUNDLAND, AND A NEW METHOD (THIN LAYER CHROMATOGRAPHY-PYROLYSIS-GAS CHROMATOGRAPHY-MASS SPECTROMETRY) FOR THEIR ANALYSIS

EDWARD D. HUDSON

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The biogeochemistry of sterols in Trinity Bay, Newfoundland, and a new method (Thin Layer Chromatography-Pyrolysis-Gas Chromatography-Mass Spectrometry) for their Analysis

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Abstract

In the context of a multidisciplinary study to determine current and past ecosystem health, sterols were analyzed in plankton, settling particles and sediments from Trinity Bay, Newfoundland by saponification, derivatization to their TMS ethers and GC and GC/MS. Plankton net tow samples and settling particles contained C_{27} and C_{28} sterols typical of marine plankton. However, higher plant C_{28} and C_{29} sterols were prominent in sediments from both in-shore and off-shore sites, indicating an appreciable terrestrial contribution to sedimentary organic carbon and either degradation or effective recycling of marine sterols. No decrease in total or individual sterols was observed down the cores, suggesting good overall preservation. The fecal sterol coprostanol was not detected in offshore sediments, net tow material or settling particles, and was present only at low levels in certain in-shore sedimentary horizons. This suggests that sewage discharges in rural Newfoundland are being efficiently degraded or dispersed.

Total free sterols in the samples were determined by Iatroscan TLC-FID on Chromarods, a widely-used method which effectively separates and quantifies lipid classes but provides no further information on the species in each class. Thus, a new method (TLC-Pyrolysis-GC/MS) was developed in which lipid bands are desorbed directly from the silica Chromarod surface into a GC/MS for analysis. Twelve lipid classes were either desorbed without further treatment, converted to trimethylsilyl derivatives on the Chromarod, or analyzed following *in situ* thermochemolysis with tetramethylammonium hydroxide. The

method's utility was demonstrated with lipids from settling particles, especially where TLC bands contained more than one lipid class (wax/steryl esters, acetone-mobile polar lipids). Wax esters up to C_{42} were detected, with alkyl and acyl distributions consistent with a zooplankton source. The wax ester/steryl ester band contained no more than 8% steryl esters, suggesting that their contribution to this band is minimal but that their contribution to total sterols may be significant. The free sterol carbon number distribution in settling particles as determined by TLC-Pyrolysis-GC/MS matched that as determined by individual molecular species analysis (predominantly C_{27}). By extending the scope of Iatroscan TLC-FID, the new method should have applications in many fields besides marine environmental studies *viz.* food science, biomedical science and petroleum analysis.

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List of Abbreviations

AMPL	Acetone-mobile polar lipids
ASG	Acylated steryl glycoside
BSTFA	Bis-*N,O*-(trimethylsilyl)trifluoroacetamide
DAG	Diacylglycerol
dw	Dry weight
E.I.	Electron ionization
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
FS	Free sterol
GC-FID	Gas chromatography with flame ionization detection
GC-MS	Gas chromatography/mass spectrometry
HC	Hydrocarbon
HPLC	High performance liquid chromatography
I.D.	Internal diameter
IUPAC	International Union for Pure and Applied Chemistry
MAG	Monoacylglycerol
OM	Organic matter
PL	Phospholipid
Pyr	Pyrolysis
R_f	Retention factor
RSD	Relative standard deviation (= coefficient of variation)
SCE	Steryl chlorin ester
SD	Standard deviation
SE	Steryl ester
SEt	Sterol ether
SG	Sterol glycoside
TAG	Triacylglycerol
TLC	Thin layer chromatography
TLC-FID	Thin layer chromatography with flame ionization detection
TLE	Total lipid extract
TMAH	Tetramethylammonium hydroxide
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
v/v	Volume/volume
WE	Wax ester
w/v	Weight/volume
w/w	Weight/weight

Acknowledgments

Firstly, I wish to thank my supervisors, Dr. Robert J. Helleur and Christopher C. Parrish, for their guidance, support, encouragement and patience during this project.

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Lastly, the role of the National Sciences and Engineering Research Council and the Department of Chemistry in preventing my starvation and eviction is gratefully acknowledged.

Dedication

Dedicated to the memory of Virginia Catherine Augemari (1972-1994).

1. Introduction

1.1 Lipids in the marine environment

Lipids are a broad class of biomolecules which are generally operationally defined as being poorly soluble in water, but soluble in non-polar organic solvents such as chloroform, although some authors prefer the narrower definition of "fatty acids and structurally or biosynthetically related compounds" (Christie, 1989), a definition which would, for example, exclude polycyclic (polynuclear) aromatic hydrocarbons and sterols. A wide variety of lipid classes are found in every compartment of the marine environment: in organisms, in the sea-surface microlayer, adsorbed onto or incorporated into settling particulates, in ancient or recent sediments, and in water in dissolved or colloidal form. They include (in order of elution on silica: Parrish, 1988) aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), wax esters, short chain (e.g., methyl) esters, acylated glycerol ethers, triacylglycerols, free fatty acids, aliphatic alcohols, sterols, diacylglycerols, monoacylglycerols, glycoglycerolipids, pigments, and phospholipids. Fig. 1.1 gives typical examples of some of these lipid classes. The functions of lipids in organisms (Zubay, 1993) range from energy capture and storage, to buoyancy control, insulation, structural components (especially of cell membranes), and vitamin and hormonal roles.

1.2 Lipid nomeclature

Given the variety of lipids found in marine material, an understanding of their

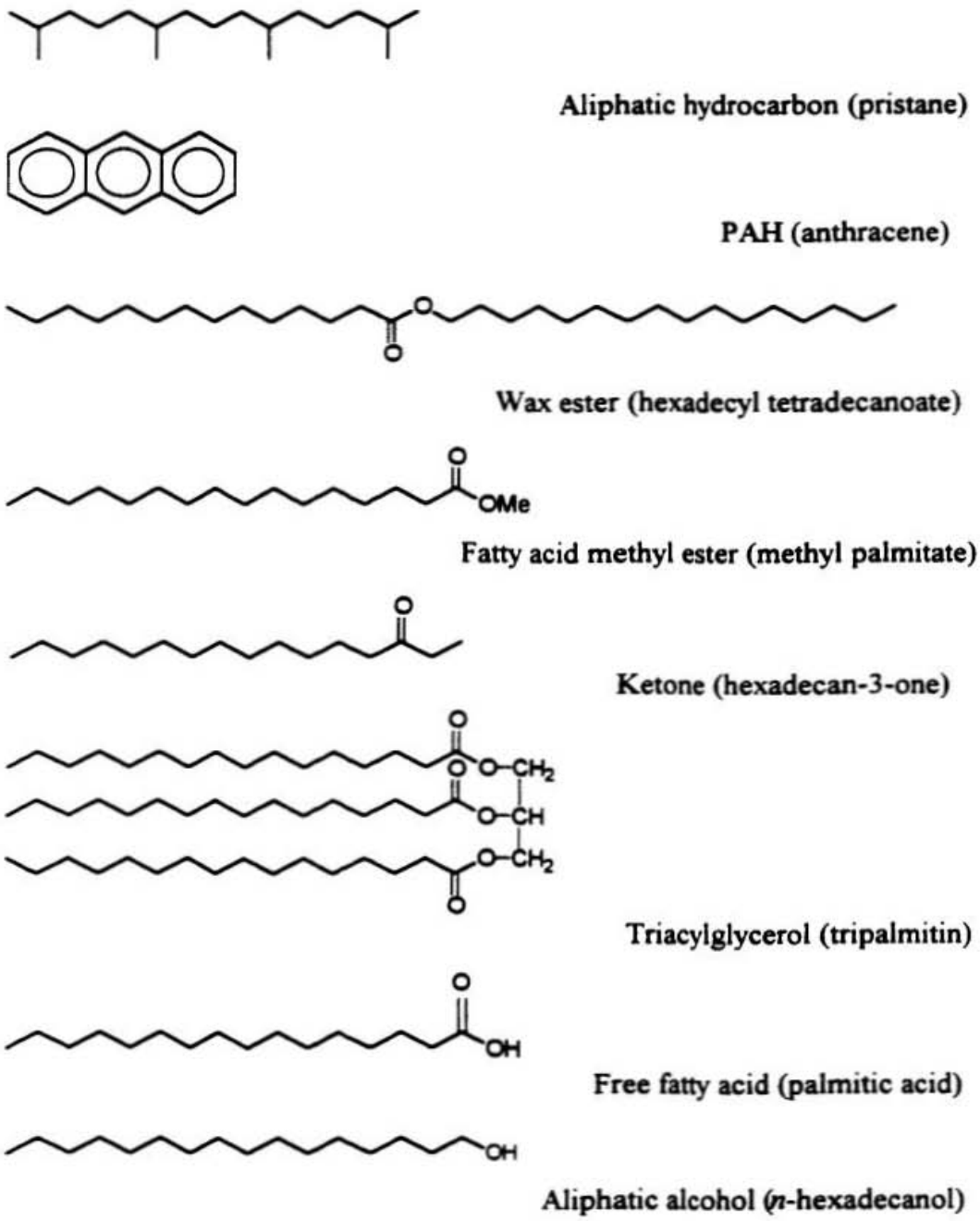
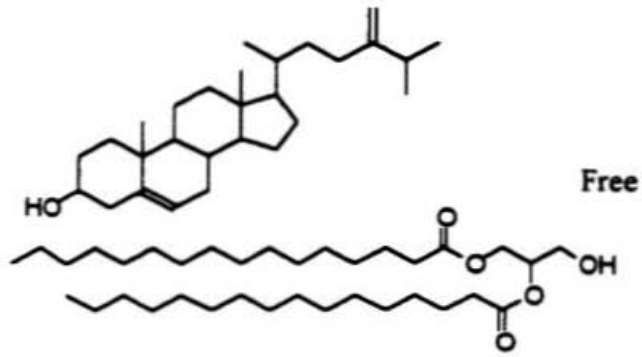
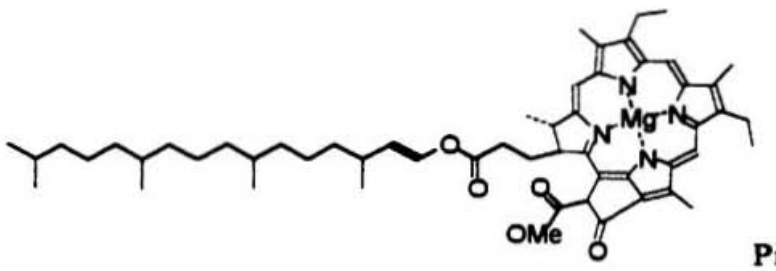


Figure 1.1 Classes of marine lipids and their structures. After Parrish (1988).

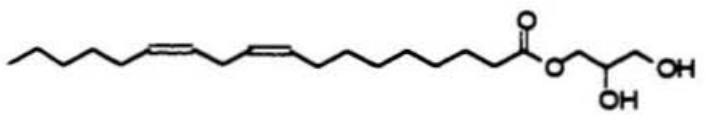


Free sterol (24-methylenecholesterol)

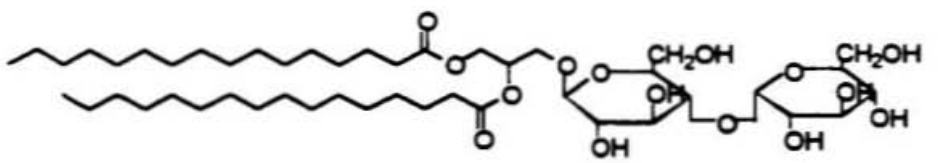
Diacylglycerol (1,2-dipalmitin)



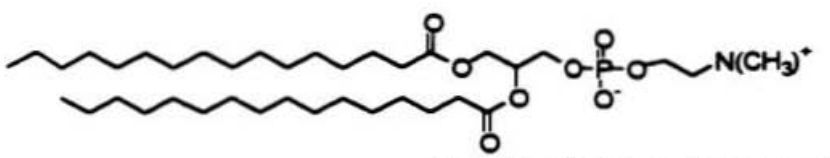
Pigment (chlorophyll *a*)



Monoacylglycerol (1-monolinolein)



Glycoglycerolipid (1,2-dipalmitoyl-3-(β1'-6-galactosyl)-galactosylglycerol)



Phospholipid (1,2-dipalmitoylphosphatidylcholine)

Figure 1.1 (continued)

nomenclature is an essential prerequisite to any discussion of their analysis, occurrence and biogeochemistry. The systems used for naming the lipid classes studied here will be briefly introduced.

1.2.1 Sterols

Sterols are formed in nature by the cyclization of (3*S*)-2,3-oxidosqualene (squalene epoxide) (Catell and Ceruti, 1991) and modification of the resulting tetracyclic molecule to give a wide variety of structures, having in common the perhydrocyclopentanophenanthrene "steroid nucleus" with rings A-D generally joined *all-trans* (Fig. 1.2a) (Zubay, 1993). In the conventional representation of the steroid nucleus (Fig. 1.2b), this absolute stereochemistry (that of cholesterol) is assumed unless another is shown. A thorough discussion of sterol nomenclature is presented by Janssen *et al.* (1991). As illustrated here by dehydrodinosterol, sterols are formally named as derivatives of cholesterol, with carbons numbered as in 1.2c. The designations α and β indicate substituents below and above the plane of the steroid nucleus, as drawn. Confusingly, α and β are also used to indicate stereochemistry at C-24 on the side chain- with a C-24 methyl substituent, 24α is equivalent to 24*R* sterochemistry (Goad, 1991). The terms stenol and stanol describe unsaturated and saturated species, respectively, although in a biogeochemical context they may refer to analogous pairs of Δ-5 unsaturated and saturated structures, in which the side chain is still unsaturated. Cholesterol itself is therefore cholest-5-en-3β-ol and dehydrodinosterol is 4,23,24-trimethylcholesta-5,22(*E*)-dien-3β-ol. In a saturated nucleus, designation of the C-5 bridgehead as α or β

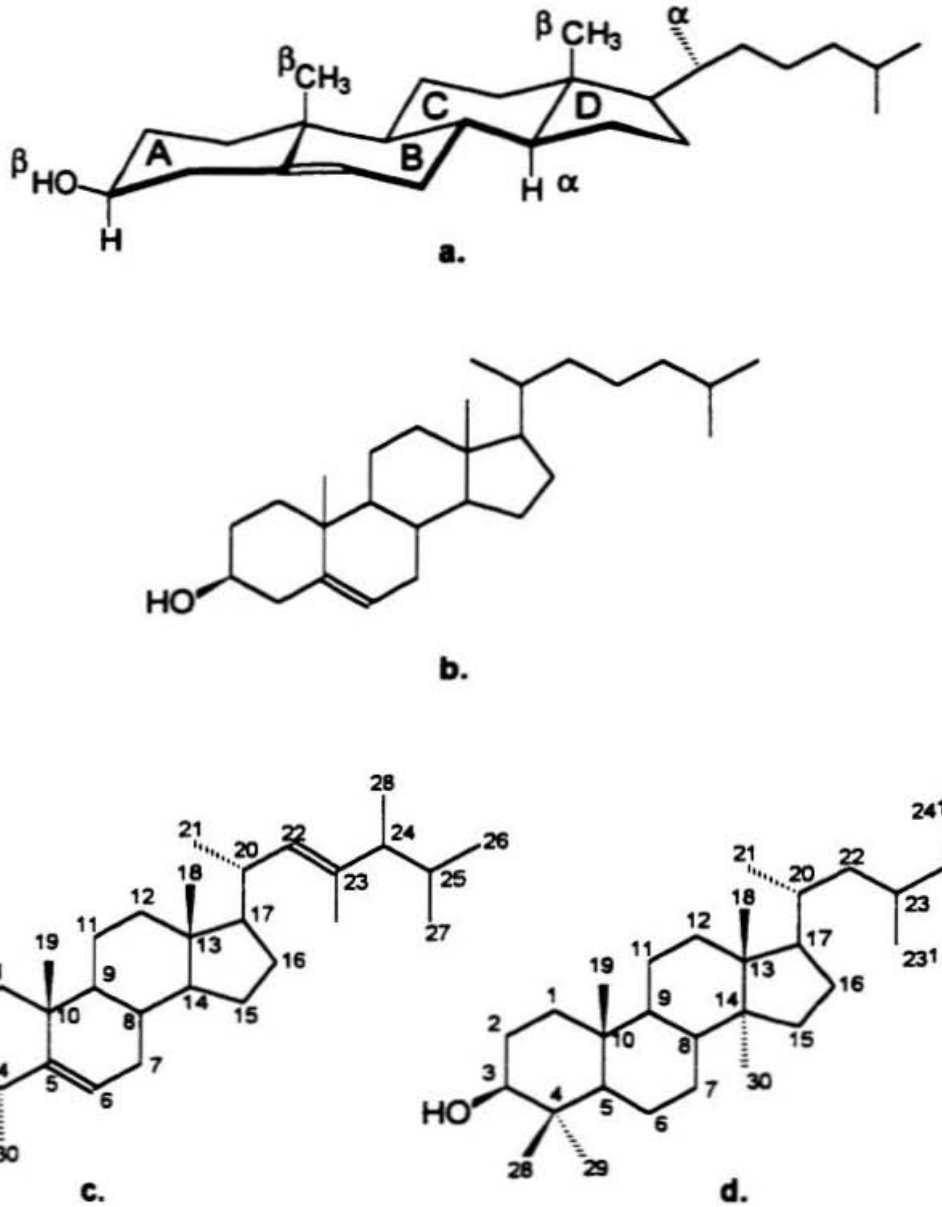


Figure 1.2. Sterol structure and nomenclature. **a**. Cholesterol, showing stereochemistry. **b.** The conventional respresentation of cholesterol. **c.** Dehydrodinosterol (4α,23,24-trimethylcholest-5,22(*E*)-dien-3β-ol). **d.** The latest IUPAC conventions for numbering substituents on the sterol nucleus.

indicates a substituent (H) below or above the plane of the steroid nucleus, respectively. Further methyl substituents (not shown) are designated C-29 (on C-28), C-31 (on C-4, β) and C-32 (on C-14, α). While 1989 IUPAC nomenclature revisions (Goad, 1991) resulted in a new numbering system (1.2d), the older conventions are still widely encountered in the literature and are therefore used here. Many sterols also have trivial names, often indicative of the source from which they were first isolated.

Abbreviation of often unwieldy sterol names is hampered by the difficulty in readily describing all aspect of a sterol's structure. The form $29\Delta^{5,24(28)E}$ may be used, this example indicating a sterol with a total of 29 carbons, with double bonds at the 5 position and between carbons 24 and 28, the latter with *E* configuration. Stereochemical considerations (α or β) can be designated thus: 3β,5β-27Δ^0 for 5β-cholestan-3β-ol (coprostanol) (Δ^0 indicating a saturated species). However, the difficulty of identifying the position of substituents on, or deletions from (indicated by the prefix "*x*-nor", where carbon atom number *x* is deleted), the cholesterol parent still remains. Many abbreviation systems are either insufficiently precise to be universal (e.g., Li *et al.* 1995) or are precise but arbitrary, giving little intrinsic indication of the structure (e.g., Patterson, 1991). Some authors (e.g., Mayzaud *et al.*, 1989) use the trivial name of one particular sterol C-24 epimer to represent either epimer, since the two are often not separated in biogeochemically-oriented works. Thus, "sitosterol" would designate both sitosterol (24α-ethylcholest-5-en-3β-ol) and clionosterol (24β-ethylcholest-5-en-3β-ol), an approach which is valid as long as it is explicitly stated. The naming system used here (Table 1.1) designates the position of substituents (or deletions) with respect to

Table 1.1. Sterol names used in the text. Letter/number designations (bold text) following the abbreviation refer to Fig. 1.3

Trivial name (Abbreviation)	*Systematic name*
cis-22-dehydrocholesterol ($27\Delta^{5,22Z}$) **A7**	cholesta-5,22(*Z*)-dien-3β-ol
cholestanol ($27\Delta^{0}$) **C1**	5α-cholestan-3β-ol
cholesterol ($27\Delta^{5}$) **A1**	cholest-5-en-3β-ol
coprostanol (5β-$27\Delta^{0}$) **B1**	5β-cholestan-3β-ol
dehydrodinosterol (4α,23,24triMe-$30\Delta^{5,22E}$) **A14**	4α,23,24-trimethylcholest-5,22(*E*)-dien-3β-ol
desmosterol ($27\Delta^{5,24}$) **A5**	cholesta-5,24-dien-3β-ol
dimethyldehydrocholestanol (4α,24diMe-$29\Delta^{22E}$) **D19**	4α,24-dimethyl-5α-cholest-22(*E*)-enol
dinosterol (4α,23,24triMe-$30\Delta^{22E}$) **D14**	4α,23,24-trimethyl-5α-cholest-22(*E*)-en-3β-ol
brassicastanol* (24Me-$28\Delta^{22E}$) **C9**	24-methyl-5α-cholest-22(*E*)-en-3β-ol
brassicasterol* (24Me-$28\Delta^{5,22E}$) **A9**	24-methylcholesta-5,22(*E*)-dien-3β-ol
*epi*coprostanol (3α,5β-$27\Delta^{0}$) **F1**	5β-cholestan-3α-ol
ethylcholesta-5,22*E*-dienol (24Et-$29\Delta^{5,22E}$) **A11**	24-ethylcholesta-5,22(*E*)-dien-3β-ol
ethylcholest-22*E*-enol (24Et-$29\Delta^{22E}$) **C11**	24-ethyl-5α-cholest-22(*E*)-en-3β-ol

Table 1.1. (Continued)

ethylcholestanol (24Et-29Δ^{0}) **C3**	24-ethyl-5α-cholestan-3β-ol
ethylcholesterol (24Et-29Δ^{5}) **A3**	24-ethylcholest-5-en-3β-ol
ethylcoprostanol (24Et-5β-29Δ^{0}) **B3**	24-ethyl-5β-cholestan-3β-ol
fucostanol (24Et-29$\Delta^{24(28)E}$) **C13**	24-ethylcholest-24(28)(*E*)-en-3β-ol
fucosterol (24Et-29$\Delta^{5,24(28)E}$) **A13**	24-ethylcholesta-5,24(28)(*E*)-dien-3β-ol
isofucostanol (24Et-29$\Delta^{24(28)Z}$) **C12**	24-ethylcholest-24(28)(*Z*)-en-3β-ol
isofucosterol (24Et-29$\Delta^{5,24(28)Z}$) **A12**	24-ethylcholesta-5,24(28)(*Z*)-dien-3β-ol
lanosterol (4,4,14triMe-30$\Delta^{8,24}$) **E5**	4,4,14-trimethyl-5α-cholesta-8,24-dien-3β-ol
24-methylcholestanol (24Me-28Δ^{0}) **C2**	24-methyl-5α-cholestan-3β-ol
4-methylcholestanol (4αMe-28Δ^{0}) **D1**	4α-methyl-5α-cholestan-3β-ol
24-methylcholesterol (24Me-28Δ^{5}) **A2**	24-methylcholest-5-en-3β-ol
24-methylenecholestanol (24Me-28$\Delta^{24(28)}$) **C10**	24-methylcholest-24(28)-en-3β-ol
24-methylenecholesterol (24Me-28$\Delta^{5,24(28)}$) **A10**	24-methylcholesta-5,24(28)-dien-3β-ol
24-nordehydrocholestanol (24nor-26Δ^{22E}) **C4**	24-nor-5α-cholesta-22(*E*)-en-3β-ol

Table 1.1. (Continued)

24-nordehydrocholesterol (24nor-26$\Delta^{5,22E}$) **A4**	24-norcholesta-5,22(*E*)-dien-3β-ol
occelasterol (24Me-27nor-27$\Delta^{5,22E}$) **A8**	24-methyl-27-norcholesta-5,22(*E*)-dien-3β-ol
trans-22-dehydrocholestanol (27Δ^{22E}) **C6**	5α-cholesta-22(*E*)-dien-3β-ol
trans-22-dehydrocholesterol (27$\Delta^{5,22E}$) **A6**	cholesta-5,22(*E*)-dien-3β-ol

* In this work, brassicasterol and brassicastanol were not separated from their 24β epimers (*epi*brassicasterol *and epi*brassicastanol, respectively). Hereafter, "brassicasterol" thus refers to both brassicasterol and *epi*brassicasterol. Likewise, "brassicastanol" designates both brassicastanol and *epi*brassicastanol.

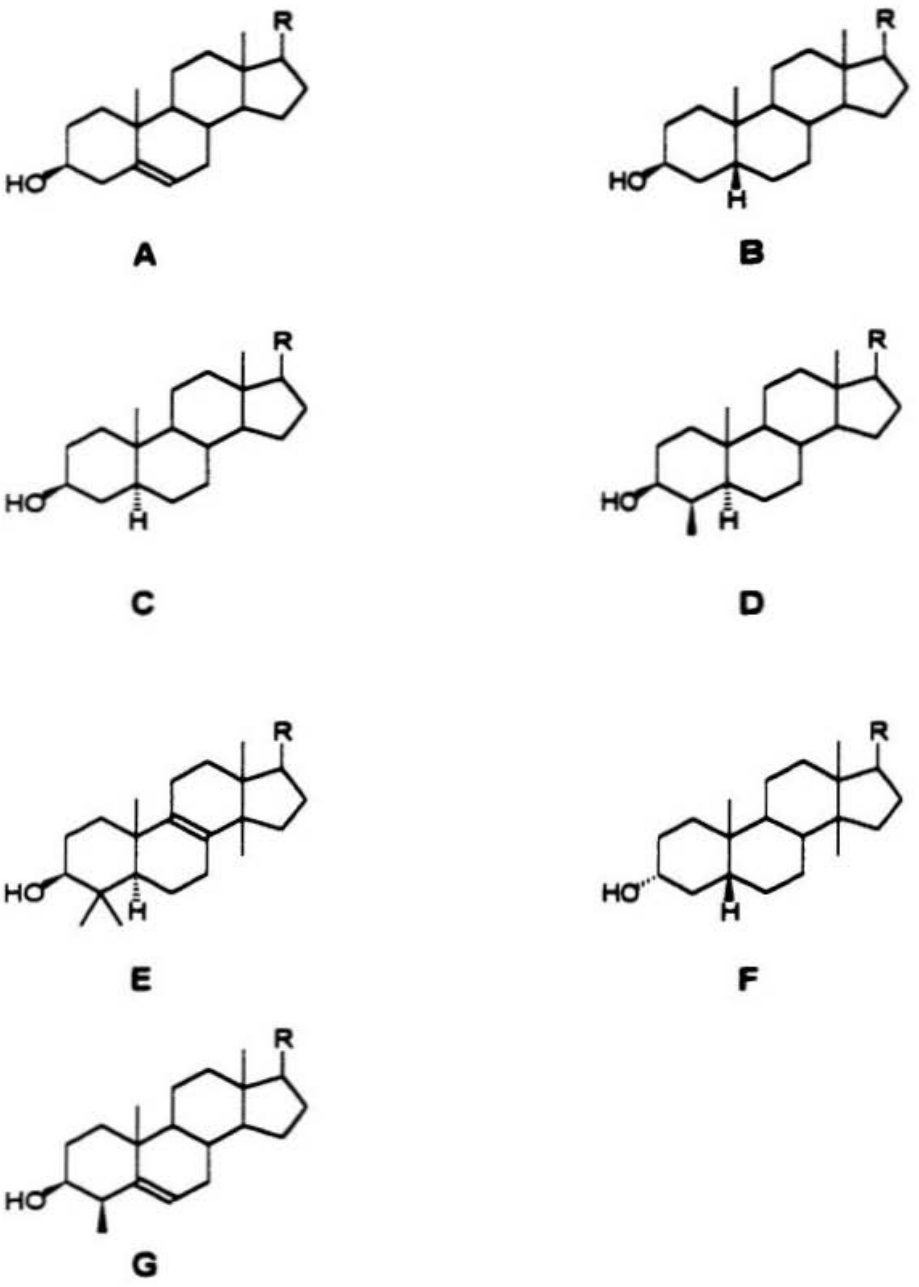


Figure 1.3. Sterol structures mentioned in the text. R = side chain (structures 1-14), Nuc = steroid nucleus (structures A-G)

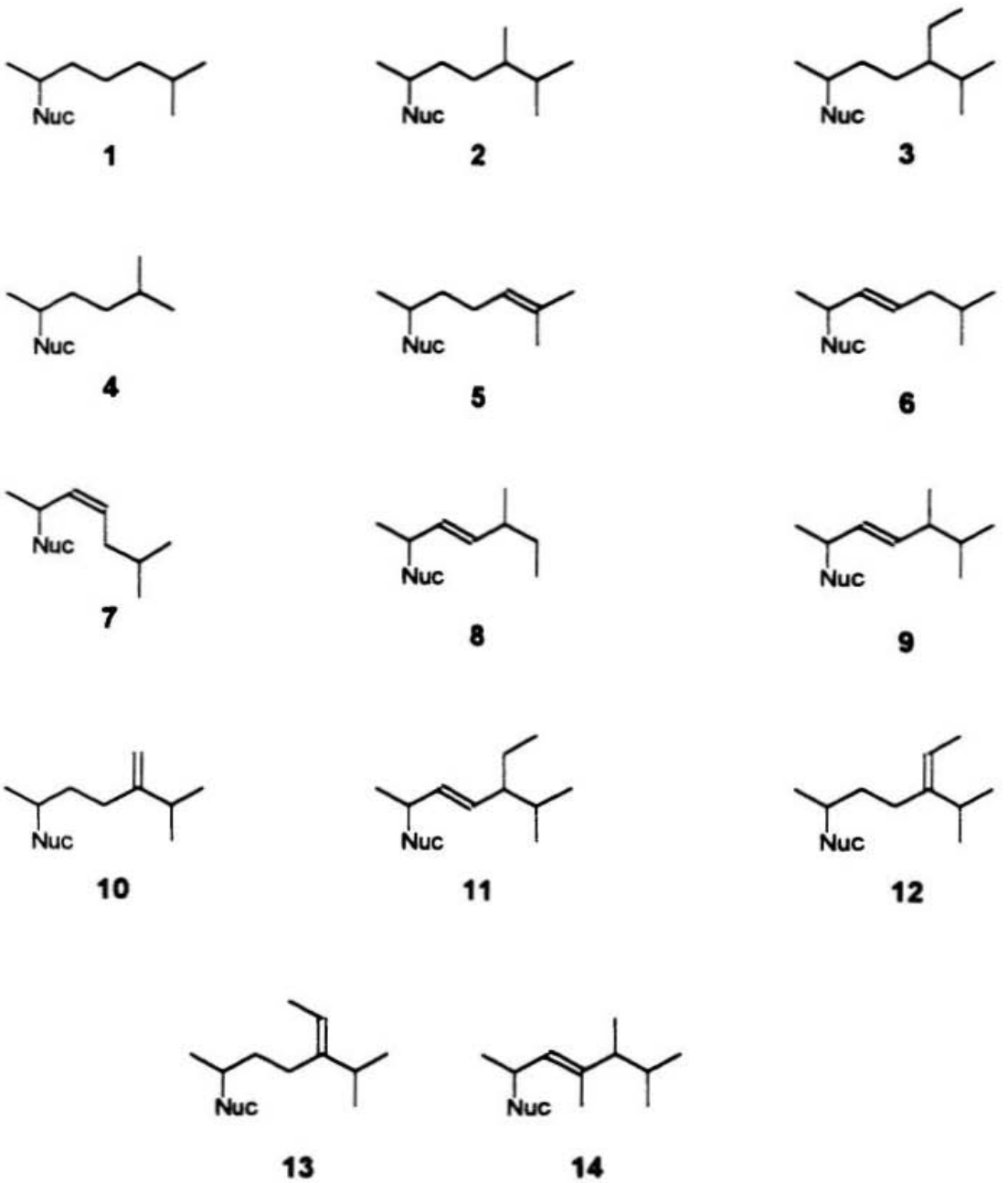


Figure 1.3. (Continued)

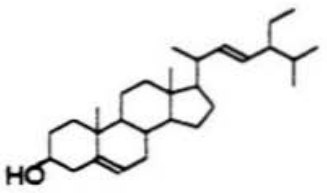
cholesterol, followed by configuration at important chiral centres, and finally the carbon number and the positions of any unsaturations. The hydroxy group is assumed to be 3β and the stereochemistry of a saturated nucleus 5α, unless otherwise stated, and C-24 stereochemistry is not designated.

1.2.2 Sterol conjugates

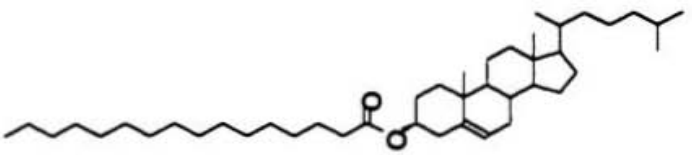
Esters of sterols with fatty acids (commonly called steryl esters, SE) are named by combining the name of the sterol and the fatty acid (the nomenclature of which is discussed below). Thus the steryl ester in Fig. 1.4 is named cholest-5-en-3β-yl hexadecanoate, or cholesteryl palmitate.

A steryl glycoside (SG) is a sterol of which the C_3 hydroxyl group is replaced by an ether (glycosidic) linkage to the C_1 position of a mono- or oligosaccharide containing up to five glycosyl residues in linear sequence (Heinz, 1996). In higher plants, a single glucose residue is the most common saccharide moiety (Wojciechowski, 1991). An acylated steryl glycoside (ASG) additionally contains a fatty acid esterified to a hydroxyl on the saccharide, most often at C_6 (Heinz, 1996). Given the variety of monosaccharides which they may contain (e.g., galactose, ramnose, mannose, xylose, ribose, glucuronic acid), the positions through which these may be linked (β1→6, β1→3, or β1→4), the nature and position of any acyl substituent, and the various sterol species themselves, the naming of specific SGs and ASGs can be complex; a detailed discussion is presented by Heinz (1996). The sterol is indicated either before (Wojciechowski, 1991; Heinz, 1996) or after (Fujino and Ohnishi,

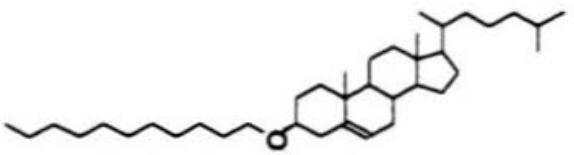
Non-polar species



Free sterol (FS)



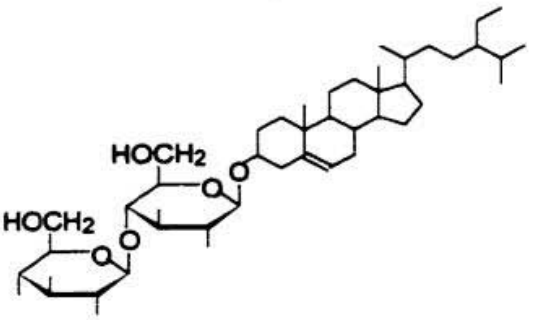
Steryl ester (SE)



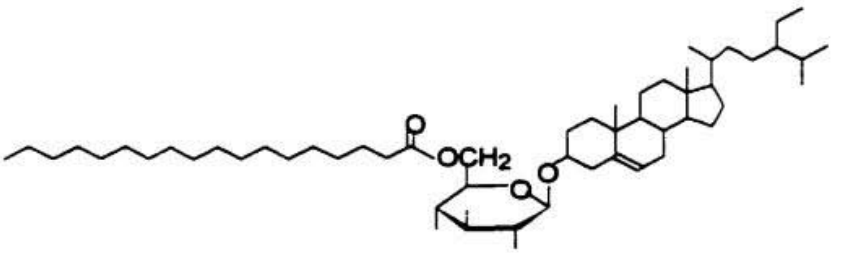
Steryl ether (SEt)

Figure 1.4. Representative structures of sterol species occurring in the marine environment.

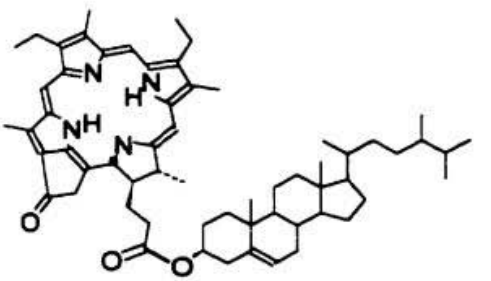
Polar species



Steryl glycoside (SG)



Acylated steryl glycoside (ASG)



Steryl chlorin ester (SCE)

Figure 1.4. (Continued)

1979) a full description of the glycosyl portion. Fig. 1.4 shows 24-ethylcholesteryl β-D-glucopyranosyl(1"→4)-β-D-glucopyranoside (SG), of which the glycosyl portion may be abbreviated to β-D-Glu(1,4)-β-D-Glu, and 24-ethylcholesteryl (6'-O-steroyl)-β-D-glucopyranoside (ASG).

Steryl chlorin esters (SCEs) (Fig. 1.4) are recently discovered sterol conjugates in which sterols are esterified to phytopheophorbide *a*, a porphyrin-like degradation product of chlorophyll (Eckhardt *et al.*, 1991). No particular nomenclature is currently specified for SCEs.

1.2.3 Fatty acids, fatty alcohols and wax esters

Individual fatty acids are often abbreviated to the form x:yωz, where x indicates the number of carbons, y the number of double bonds and z the number of carbons from the terminal (ω) methyl group (inclusive) to the first double bond. Unless otherwise indicated, all bonds are assumed to be *cis* (*Z*) in configuration and methylene interrupted. For example, octadeca-9,12-dienoic acid (linoleic acid) (Fig. 1.1) would be abbreviated to 18:2ω6. More unusual structures such as *iso*- or *anteiso*- branching (indicating a methyl group on the ω2 or ω3 carbons, respectively), or *trans* (*E*) double bond configuration, are indicated by the appropriate prefixes. Trivial names for many acids are still widely used.

Long chain aliphatic (fatty) alcohols, though not as abundant a constituent of lipid material as fatty acids, may be similarly abbreviated, as long as it is clear which class of compound is meant. Thus, wax esters, the esters of fatty acids with fatty alcohols, are here

abbreviated as x:y/x':y', indicating the number of carbons (x) and double bonds (y) in the alkyl and acyl (') portions, respectively.

1.2.4 Neutral acylglycerols

Several important lipid classes consist of glycerol (1,2,3-propanetriol) esterified to one or more fatty acids; those having one, two and three fatty acids are termed monoacylglycerols (MAGs), diacylglycerols (DAGs) and triacylglycerols (TAGs), respectively. For acylglycerols containing the same fatty acid at each position, the acid's trivial name is readily used to name the acylglycerol. Using the example of palmitic (hexadecanoic) acid, 1-monopalmitin designates the MAG 1-monohexadecanoylglycerol, 1,3-dipalmitin would be the DAG 1,3-dihexadecanoylglycerol, and tripalmitin is the TAG trihexadecanoylglycerol. The naming system used here does not designate stereochemical configuration around the glycerol C-2, since this is unimportant in this work; this issue is discussed by Perkins (1991). Where DAGs or TAGs contain two or three different fatty acids, lengthier systematic names are needed, although the trivial names of the fatty acids may be incorporated into these.

1.2.5 Phospholipids

Phosphoglycerides, the phospholipids studied here, are derivatives of glycerol-3-phosphate which may be esterified at the phosphoric acid portion as well as at both remaining hydroxyls on glycerol. They are named according to the substituent on phosphoric

acid (Zubay, 1993) and the fatty acids on the glycerol hydroxyls are indicated as prefixes. Thus, the phospholipid in Fig. 1.1 is distearoylphosphatidylcholine. Phosphatidylcholines are also known as lecithins. Other common substituents esterified to the phosphate moeity include serine, *myo*-inositol, and glycerol. If this substituent is a hydrogen atom, the lipid is termed a phosphatidic acid.

1.3 Relevance of tracing organic carbon in the marine environment.

Marine primary production by phytoplankton in surface waters underpins the entire marine food web, including commercial and subsistence fisheries. While most primary production is also consumed or remineralized in surface waters (Wakeham and Lee, 1991), an important fraction sinks through the water column and nourishes mid-water and benthic organisms. Furthermore, this sinking organic matter transports atmospheric carbon into sediments and deep ocean waters, where it may remain for centuries (Libes, 1992), thereby influencing global climate (Sarmiento and Sundquist, 1992). Therefore, an understanding of the biogeochemistry (source organisms, processes and forms, rates of production and degradation, and ultimate fate) of organic carbon in the marine environment is of vital importance.

Organic compounds from terrestrial sources which enter the marine environment in run-off or through atmospheric deposition provide an additional energy input which can be utilized and degraded by marine organisms (Libes, 1992). Human activities on land which affect this input (e.g., agriculture, deforestation) can therefore affect marine ecosystems.

Furthermore, the magnitude and dispersal patterns of terrestrial input may indicate which parts of the marine environment may be impacted by anthropogenically introduced pollutants (e.g., petroleum products).

1.4 Lipids as biomarkers in the marine environment

Lipids can provide a wealth of information about organisms and processes in the marine environment, reflecting sources of organic matter and its transformational history in the water column or sediments (Cranwell, 1982). Certain fatty acids can be traced unmetabolized through food webs. Branched fatty acids (e.g. *iso* 14:0 and *iso* 16:0, and 15:0 and 17:0 *iso* and *anteiso* acids) in sediments and estuarine particles are markers of bacterial carbon input (e.g., Scribe *et al.*, 1991). Toxic dinoflagellates and diatoms produce characteristic fatty acids (Parrish *et al.*, 1992), such as 16:4ω1 in the case of *Nitzschia pungens* (Parrish *et al.*, 1991). Moreover, lipids are often the slowest of all biomolecules to degrade in the early stages of diagenesis (Harvey *et al.*, 1995; Ishiwatari *et al.*, 1995), particularly in anoxic sediments. The use of lipids as biomarkers in this fashion is reviewed by Saliot *et al.* (1991).

In addition to using individual molecular species as biomarkers, lipid class data can yield environmental information. Goutx *et al.* (1990) obtained characteristic lipid class profiles for common marine microalgae. Furthermore, lipid class composition can indicate the physiological state of organisms. Applications have included using total triacylglycerol/sterol ratios to gauge the condition of fish, crustacean and bivalve larvae

(Fraser, 1989) and gauging the quality of halibut eggs by determining storage lipid content (Daniel *et al.*, 1993).

1.4.1 Sterols in the marine environment

Sterols are found in all eukaryotes, as well as in cyanobacteria (Patterson, 1991). Their principal function is as a membrane component, in which they modulate the fluidity of cell membranes; furthermore, they are precursors to certain vitamins and regulatory substances (Goad, 1991; Zubay, 1993). While some organisms (mammals, higher plants) can synthesize sterols *de novo*, others (e.g., crustaceans) (Serrazanetti *et al.*, 1989) can only modify dietary sterols and still others (e.g., many molluscs: Napolitano *et al.*, 1993) incorporate unmodified dietary sterols.

A wide variety of sterol structures occur in the oceans, either originating there or from input from terrestrial systems (see Volkman, 1986). Brassell and Eglinton (1981) isolated 69 different sterols from sediments from the Japan trench, and the identification of 30 or more sterols in settling particles in various marine environments (Smith *et al.*, 1983; Bayona *et al.*, 1989; Harvey and Johnston, 1995) is not unusual. These figures are not surprising considering the variety of organisms producing these sterols, with up to 74 sterols having been isolated from a single organism (Itoh *et al.*, 1983), and the variety of processes which may subsequently transform these (reduction, dealkylation, etc.) (Cranwell, 1982).

Most organisms which synthesize C-24 alkylated sterols produce only one C-24 epimer, although a few terrestrial plants, including certain Cucurbitaceae and Crassulaceae such as *Kalanchoe pinnata* (air plant) (Akihisa *et al.*, 1991b) synthesize both C-24 epimers

of a particular structure. Generally, higher plants will contain the 24α epimer (e.g., 24α-ethylcholesta-5,22(*E*)-dien-3β-ol, stigmasterol), while algae contain the 24β epimer (e.g., 24β-ethylcholesta-5,22(*E*)-dien-3β-ol, poriferasterol) (Maxwell *et al.*, 1980).

Sterols may occur in aquatic environments as free sterols, or in steryl esters (Wakeham and Frew, 1982), steryl ethers (Boon and DeLeeuw, 1979), steryl glycosides or acylated steryl glycosides (Gladu *et al.*, 1991; Veron *et al.*, 1996), or steryl chlorin esters (Eckardt *et al.*, 1991; Harradine *et al.*, 1996), the last formed during zooplankton herbivory. In some environments the sterol composition in these forms differs from that of the free sterols. Sedimentary steryl esters in Loch Clair, UK, had a lower stanol/Δ^5-stenol ratio than free sterols in the same sediment (Cranwell, 1982). Conversely, in settling particles in the equatorial Atlantic, free sterol and steryl ester molecular distributions were well correlated (Wakeham *et al.*, 1980), and sedimentary steryl chlorin esters may be a better indicator of the sterol composition of source organisms than free sterols themselves (Pearce *et al.*, 1998).

1.4.1.1 Sterols as biogeochemical markers

Even among lipid classes, sterols are some of the best biogeochemical marker compounds, due to their resistance to degradation (Saliot *et al.*, 1991; Quemeneur and Marty, 1992) and their wide variety of structures. Intact sterols have even been found in lignite coals (Del Rio *et al.*, 1992)

The sterol profile of diatoms can be characteristic of a particular class, family, genus or even species (Barrett *et al.*, 1995); 23,24-dimethylcholest-5,22*E*-dien-3β-ol may be a

unique marker for the widespread *Fragillaria pinnata*. Table 1.2 indicates certain sterol structures broadly associated with various algal taxa, although there are many exceptions. Trends include the predominance of fucosterol in most brown algae (Phaeophyceae) and of cholesterol in many red algae (Rhodophyceae), with desmosterol also frequently present in the latter. 4α-Methyl sterols are most widespread among the dinoflagellates (Dinophyceae), and 24-methylenecholesterol and (*epi*)brassicasterol are frequently found in diatoms (Bacillariophyceae).

Table 1.2. Characteristic sterols of various algal taxa (after Patterson, 1991)

Algal class	*Principal or characteristic sterols*
Dinophyceae (dinoflagellates)	4-Methyl sterols, e.g., dinosterol; cholesterol
Prymnesiophyceae	Δ^5 sterols; (*epi*)brassicasterol
Phaeophyceae (brown algae)	Fucosterol
Baccilariophyceae (diatoms)	Varies; many Δ^5 and C_{28} sterols
Rhodophyceae (red algae)	Cholesterol, desmosterol
Chlorophyceae (green algae)	Varies; Δ^5 sterols, some Δ^7 or $\Delta^{5,7}$ sterols; C_{29} sterols
Xanthophyceae	Ethylcholesterol
Charophyceae	Ethylcholesterol

The use of sterol biomarkers is complicated by the variability of the sterol composition which may occur even within certain algal genera (Patterson, 1991), and the possibility that algal growth conditions may alter their sterol composition (Veron *et al.*, 1996). 24-Ethylcholesterol, until recently thought to be an exclusively terrestrial sterol, is

also produced by some algae (Volkman, 1986; Patterson, 1991; Li *et al.* 1995). The sterol dinosterol was once thought to be solely produced by dinoflagellates, but has also been found in certain marine diatoms (Volkman *et al.*, 1993). Furthermore, while some invertebrates accumulate unmodified dietary sterols, others such as copepods dealkylate dietary phytosterols (Serrazanetti *et al.*, 1989), therefore modifying the sterol input into sinking particles. Nonetheless, certain patterns in the sterol composition of marine samples can provide useful biogeochemical information. A predominance of 4-methyl sterols likely indicates a significant dinoflagellate contribution. While certain algae produce predominantly C_{28} and C_{29} sterols, proportionately high abundances of these are in many cases still indicative of terrestrial input (e.g., Laureillard and Saliot, 1993). Additionally, examining the ratios of certain sterols to others (Li *et al.*, 1995), or the co-variation of certain sterols with each other (Pockington *et al.*, 1987) and with other biomarkers (Yunker *et al.*, 1995) yields further information.

1.4.1.2 Sterols as fecal biomarkers

Sewage discharges from coastal communities may have various impacts on the marine environment. They may disrupt marine life by lowering dissolved oxygen levels (Libes, 1992), foul the gills of fish and filter feeding organisms (Frankel 1995), and alter fish abundances (Mearns, 1982). Human health may also be impacted through pathogens (e.g., skin and eye infections, hepatitis A) and heavy metals in sewage being introduced into the diet through contaminated seafood (Frankel, 1995). Therefore it is important to determine the

extent and distribution of human sewage contamination, especially in areas such as Newfoundland, which has traditionally depended on marine fisheries and where there is great interest in the further development of aquaculture.

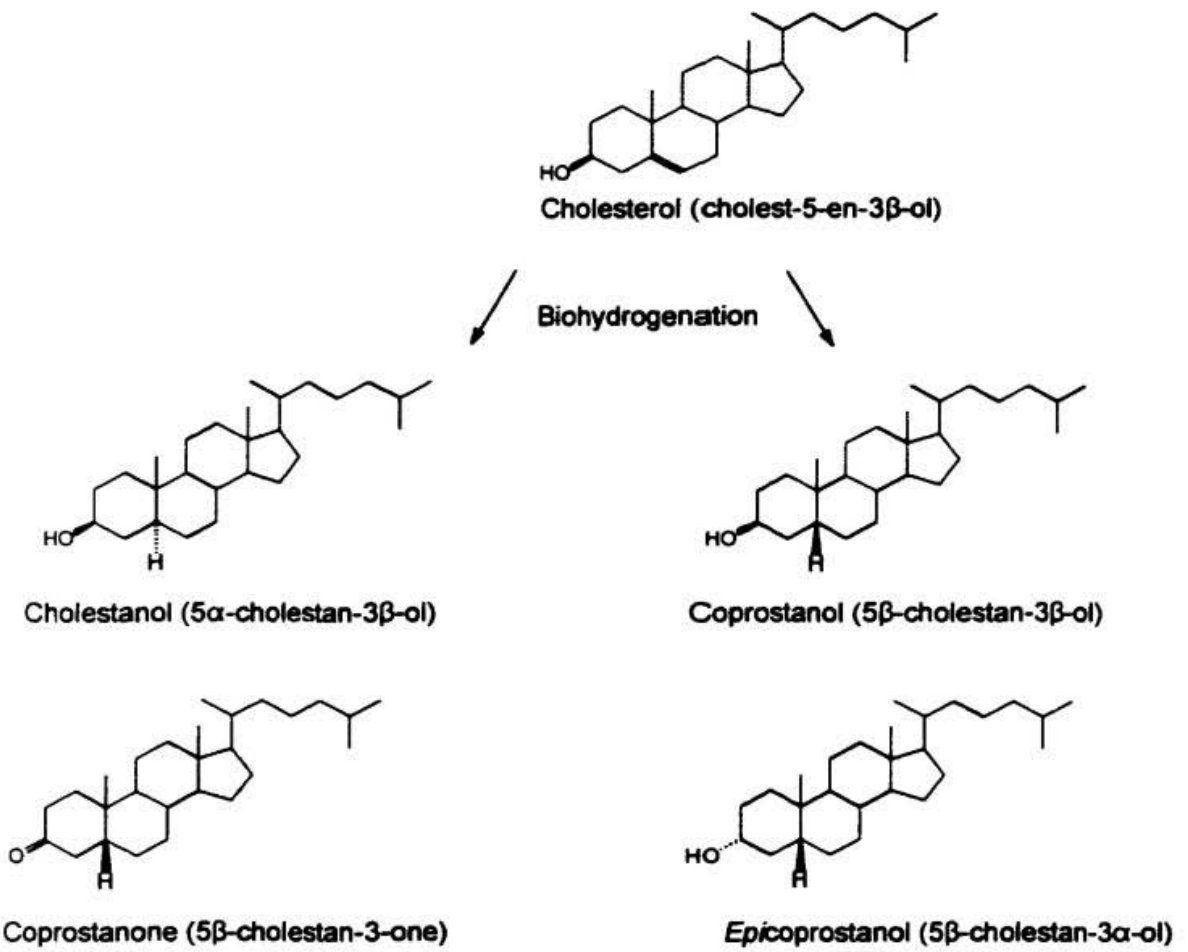


Figure 1.5. Δ^5-Hydrogenation of cholesterol can produce either cholestanol or coprostanol. Coprostanone and *epi*coprostanol are important in differentiating sewage inputs from other possible coprostanol sources.

The sterol coprostanol (5β-cholestan-3β-ol,) has long been used as a marker of sewage contamination (e.g., Hatcher and MacGillivary, 1979; Pierce and Brown, 1984; Writer *et al.*, 1995), providing an alternative to fecal coliform counts, which may be unreliable due to the variability in coliform survival in different environments (Nichols and Espey, 1991). Bacterial Δ^5-hydrogenation of dietary cholesterol in the intestines of mammals produces this sterol rather than its isomer, cholestanol (5α-cholestan-3β-ol) (Fig. 1.5) and its distribution in river, harbour and coastal sediments (e.g., Writer *et al.*, 1995; Venkasetan and Kaplan, 1990) in beach sands and greases, and in the sea surface microlayer (Nichols *et al.*, 1996) has therefore been widely studied.

Pocklington *et al.* (1987) have suggested that coprostanol may not be an unambiguous fecal biomarker, since its concentration in Bedford Basin, Nova Scotia, was closely correlated with several distinctive algal biomarkers. Furthermore, anoxic sedimentary environments appear to favor the formation of 5β-stanols over 5α-stanols from Δ^5-stenols (Meyers and Ishiwatari, 1993). Writer *et al.* (1995) used a coprostanol concentration of 0.010 μg/g dw sediment as a cut-off to indicate sewage contamination, but coprostanol concentrations far higher than this may occur in sediments from remote marine locations (e.g., Grimalt *et al.*, 1991; Colombo *et al.*, 1997), suggesting sources other than sewage. Moreover, it is necessary to differentiate fecal input due to livestock from that due to domestic sewage (Nichols *et al.*, 1996). Nonetheless, to ascertain whether human fecal contamination is present in waste waters or sediments, 5β-stanols and ketone intermediates may be determined, and their abundances compared to that of other sterols. Mudge and

Bebianno (1997) used the coprostanol/cholesterol ratio to ascertain the extent of sewage contamination in sediments. Quemeneur and Marty (1992) calculated this ratio and the 24-ethylcoprostanol/sitosterol ratio as indicators of fresh domestic wastewater. Other biomarkers, such as the 18:1ω11/18:1ω9 fatty acid ratio (Quemeneur and Marty, 1992) can corroborate evidence of sewage input. The ratio of coprostanol to coprostanol plus cholestanol, 5β/(5α + 5β), has also been used (Grimalt *et al.*, 1990; Li *et al.*, 1995; Mudge and Bebianno, 1997), as has the ratio of coprostanol to dinosterol (Venkantesan and Kaplan, 1990). The *epi*coprostanol to coprostanol ratio can distinguish the feces of certain marine mammals from that of humans (Venkantesan and Santiago, 1989), although *epi*coprostanol is also prevalent in sewage treated in sludge digestors (Grimalt *et al.*, 1990). Lastly, the Δ^4-stenone and stanone intermediates in stanol formation may be examined.

1.5 Methods for lipid analysis

1.5.1 Sterol analysis

While individual sterols may be separated and analysed by HPLC (Akihisa *et al.*, 1991a), GC is the most commonly used chromatographic method for their analysis. The free alcohols may be chromatographed (e.g., Mayzaud *et al.*, 1989; Quemeneur & Marty, 1992), but most methods precede GC with some form of derivatization. Conversion to less polar derivatives allows convenient analysis on common non-polar stationary phases, on which the free alcohols tend to tail excessively. Sterol acetates may be prepared (e.g., Wakeham *et al.*, 1980; Itoh *et al.*, 1982; Conway and McDowell Capizzo, 1991) but trimethylsilyl ethers are

by far the most commonly used derivatives for marine samples, and many minor variations in their preparation have been reported, along with reviews of relative retention times and mass spectra (Jones *et al.*, 1994). Where the free alcohols are analyzed, their prior isolation by silica column chromatography, preparative TLC (e.g., Mayzaud *et al.*, 1989) or HPLC (e.g., Quemeneur & Marty, 1992) may be necessary, although many studies employ this approach anyway, since it increases the certainty that mainly sterols will appear in the GC chromatogram (e.g. Venkatesan *et al.*, 1987; Colombo *et al.*, 1996; Laureillard and Saliot, 1993). Such pre-fractionation may also be used to isolate specific sterol subclasses, such as 5β-stanols (Bull *et al.*, 1998), or to separate 4,4-dimethyl, 4-monomethyl and 4-desmethyl sterols (Volkman, 1986).

A notable shortcoming of most GC methods for sterols is their inability to separate C-24 epimers. Such separation has been achieved on long capillary columns (100 m or more) (Maxwell *et al.*, 1980; Thompson *et al.*, 1981), but analysis times are too long (3-10 hrs) for routine use. Reverse-phase HPLC is useful for separating specific pairs of C-24 epimers (Akihisa *et al.*, 1991b), and ^{1}H nuclear magnetic resonance (NMR) (e.g., Goad, 1991) and X-ray crystallography (Ling *et al.*, 1970) are able to distinguish them, although these require isolation of significant quantities of the individual sterol. Due to sample unavailability and complexity, C-24 epimers are rarely distinguished in marine biogeochemical studies, despite the potential to differentiate algal and higher plant inputs by C-24 stereochemistry.

Steryl esters are not differentiated from free sterols by common methods which begin

with saponification (base hydrolysis) of the total lipid extract. However, they may be analyzed intact by GC or GC-MS (Wakeham & Frew, 1982; Bull *et al.*, 1998), although high temperatures are required and analysis times are typically long. Steryl ethers can be similarly analyzed (Boon and DeLeeuw, 1979). Polar sterol species present the greatest analytical challenges. Base hydrolysis does not release sterols from steryl glycosides or acylated steryl glycosides, and they are therefore not detected by conventional methods for marine sterol analysis which generally involve alkaline saponification. Fully trimethylsilylated steryl glucosides have been analyzed by GC on a short packed column (Laine and Elbein, 1971). Reverse-phase HPLC may be used, although detection is problematic (Heinz, 1996). The acid hydrolysis used by some workers (e.g., Veron *et al.*, 1996) to release sterols from these polar conjugates may alter the structure of those sterols (Heinz, 1996); periodate cleavage of the ether linkage is preferable.

1.5.2 Lipid class analysis

As previously noted, a preparative lipid class separation may be a useful precedent to molecular species analysis of an individual class. Often, quantitation by lipid class is the desired result. While HPLC has been used for this (Gonzalez-Castro *et al.*, 1996), most workers have used some form of TLC.

1.5.2.1 Iatroscan TLC-FID

In the past fifteen years, Iatroscan thin layer chromatography-flame ionization

detection (TLC-FID) has become a widely accepted technique for lipid class analysis. The analytical system consists of 15-cm quartz rods (Chromarods), 0.9 mm in diameter, coated with a 75-μm thick layer of porous silica gel particles (5-μm diameter) sintered into a glass frit (Ackman *et al.*, 1990). The sample is spotted at one end of the rod and developed as in plate TLC. It is then passed through a flame ionization detector in the Iatroscan instrument, where each separated band is quantified by combustion. Up to ten rods can be scanned simultaneously, providing high sample throughput. Partial scanning of the rods, and the further development of uncombusted material, allows multidimensional separation and detection impossible in conventional plate TLC.

Applied to marine lipid classes, Iatroscan TLC-FID can distinguish energy storage classes, pollutants, membrane lipids, or indicators of organic matter degradation (e.g., Goutx *et al.*, 1990). The technique is especially useful for analyzing such classes as phospholipids (Hazel, 1985; Gérin and Goutx, 1993) and pigments which are too involatile or polar for GC (Volkman and Nichols, 1991); it has also been applied to the rapid determination of total lipid carbon (Harvey and Patton, 1981; Ogura *et al.*, 1987) and in other methods as a support for quantitation rather than a separation medium *per se* (Kramer *et al.*, 1991). Like conventional TLC plates, the silica Chromarod coating can be impregnated with various modifiers, for example, copper (II) sulfate to increase FID response and improve detection uniformity (Kaimal and Shantha, 1984), oxalic acid to efficiently separate phospholipid types (Banerjee *et al.*, 1985), boric acid to separate isomeric acylglycerols (Tanaka *et al.*, 1980; Tatara *et al.*, 1983), or silver (I) nitrate to separate lipids by degree and/or geometry of

unsaturation (Sebedio and Ackman, 1981; Sebedio *et al.*, 1985). Outside of lipid analysis, applications of TLC-FID have included quantitation of the aliphatic, aromatic, resin and ashphaltene fractions of crude oils (Karlsen and Larter, 1991) and analysis of polystyrene and polyisoprene polymers (Takenaka *et al.*, 1995).

The principal drawback of Chromarod TLC, indeed TLC generally, is its inability to resolve individual closely-related molecular species in complex samples. As noted above, molecular species analysis of a particular lipid class is usually done by GC (Kuksis and Myher, 1989) or HPLC (Kuksis *et al.*, 1991), often requiring pre-fractionation, saponification, derivatization or other treatment (Wood, 1991). A method allowing individual compounds within a band on a Chromarod to be analyzed further would increase the amount of information available from a lipid class separation.

Sterols, in particular, illustrate the difficulties in TLC-FID as currently practiced. As noted above, they occur in the marine environment in several different forms of varying polarities. In a typical Chromarod lipid class separation used in our laboratory (Parrish, 1987), sterol species appear in several different bands. Free sterols (FS) are generally resolved into a pure band after development in the second solvent system, although some contamination of this band by 1,3-diacylglycerols is possible. Steryl esters (SE) co-elute with wax esters. Lastly, any steryl glycosides (SG) or acylated steryl glycosides (ASG) could be expected to elute in the third development, although their R_f values have never been determined. It is expected that steryl chlorin esters would also elute in this region.

1.5.3 Coupling of TLC with GC or MS

Various methods have been reported to couple the rapid, inexpensive, low-resolution TLC separation directly to further separation or accurate quantitation (GC), or superior specificity and identification (mass spectrometry, MS). Direct transfer from TLC to mass spectrometry has been accomplished by Xe^+ bombardment to remove phospholipids from TLC plates as secondary ions (Kushi and Handa, 1985), and by laser desorption of TLC-separated simple mixtures of polypeptides and dyes (Gusev *et al.*, 1995), various quinones, alcohols, acids and aromatic esters (Ramaley *et al.*, 1985), and the drug Naproxen (Fanibanda *et al.*, 1994). Laser desorption has also been used to transfer pesticides from TLC onto a GC column (Zhu and Yeung, 1989). Lyle and Tehrani (1981a, b) reported the introduction of polymers and water-soluble vitamins from cut TLC plates into a gas chromatograph by pyrolysis, although the GC step was used primarily for accurate quantitation (already possible in TLC-FID) rather than for further separation, especially since the pyrolytic introduction led to multiple products from a single substance on the TLC plate. However, all of these methods have been developed for plate TLC rather than to complement the specific advantages of Chromarods, particularly in lipid analysis. Furthermore, they rely on TLC as the principal separation step for individual molecular species, rather than taking advantage of the broad profiling capabilities of TLC for complex, naturally occurring mixtures.

1.6 Objectives

1.6.1 Sterols in Trinity Bay, Newfoundland

The collapse of Newfoundland groundfish stocks, with its devastating economic and social impacts, provided an impetus for the multi-disciplinary Eco-Research program. This three-year study, funded under Environment Canada's "Green Plan", aimed to examine factors contributing to sustainability in Newfoundland as a representative North Atlantic cold-ocean ecosystem and society. Within this framework, the Marine Team, a sub-unit of the Eco-research program comprising 7 workers in Memorial's Chemistry and Earth Sciences departments and the Ocean Sciences Centre, aimed to gauge ecosystem health by examining specific sets of biomarkers (various lipids, phenolic compounds) to determine sources, fates and transformations of organic matter in the marine environment, and the relative contributions of marine *vs.* terrestrial and natural *vs.* anthropogenic input (Section 1.3). This aspect of the study focused on the western Trinity Bay region (Fig. 1.6).

In rural Newfoundland, sewage is typically disposed of in bays and harbours with little or no pre-treatment. The potential use of 5β-stanols as a fecal markers for this input initially motivated the study of sterols in marine samples from the region. In conjunction with other lipid biomarker studies of ecosystem health (Budge and Parrish, 1998; Favaro, 1998; Parrish, 1998b), the study also aimed to use sterols to identify sources and transformations of organic matter in marine environment of western Trinity Bay, as well as ascertain whether sewage inputs were recognizable in the current ecosystem or in the region's sedimentary record.

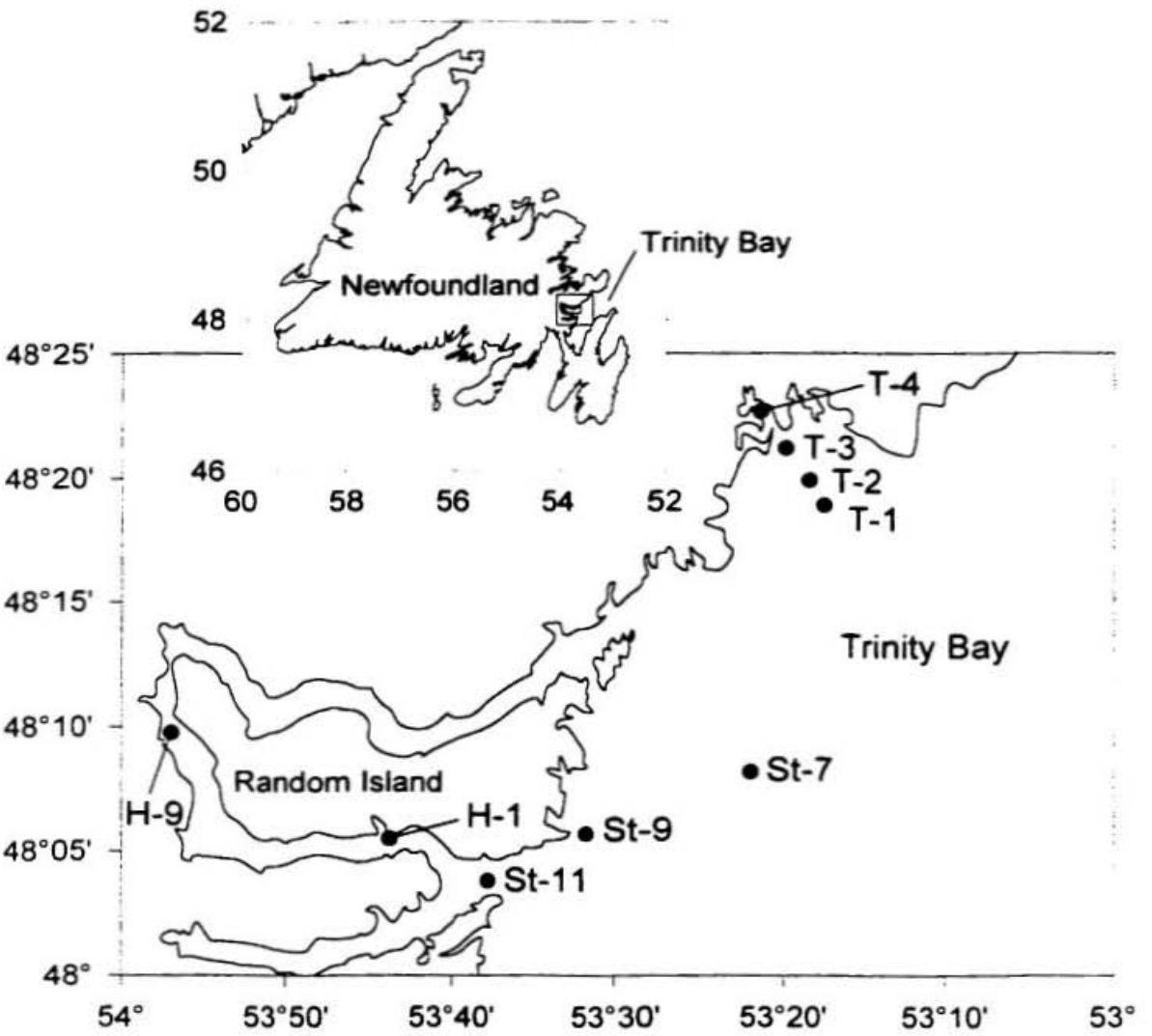


Figure 1.6. Study area and sampling locations in western Trinity Bay, Newfoundland.

1.6.2 Lipid analysis by TLC-Pyrolysis-GC/MS

The second part of this work aimed to extend the scope of Chromarod TLC by thermally desorbing the lipid class bands directly onto a GC column for further analysis of their constituents by GC-MS. As well as providing further information on molecular species within each separated class, this would clarify the purity and composition of the bands being concomitantly quantified by FID scanning, especially for those bands which group several classes of lipids, such as the steryl ester/wax ester band and the so-called acetone-mobile polar lipids (AMPL), a band including glycoglycerolipids, pigments and monoacylglycerols. Furthermore, the work aimed to develop the technique such that it would be of general utility for a wide range of lipid classes, using widely available columns and avoiding extremely high elution temperatures and long elution times.

The effort was targeted at sterol-containing bands in Chromarod separations, in conjunction with the first part of this work. However, other bands, particularly acyl lipid bands, are also of interest due to their role as energy storage compounds in marine organisms. The new technique would be applicable not only to the lipid biogeochemical studies conducted in the framework of the Eco-Research program, but also to other such studies and those utilizing Iatroscan TLC-FID in food science, biomedical science and aquaculture nutrition studies.

Part I. Biogeochemistry of sterols in plankton, settling particles and sediments in Trinity Bay, Newfoundland

2. Experimental

2.1 Chemicals, glassware and instrumentation

All glassware used was heated at 450 °C overnight or, where this was not possible, rinsed at least three times with the solvent to be used before use. Initially, all solvents used for rinsing and for dilution were trace analysis grade (Omnisolv (BDH) or Optima (Fisher)). However, blank 1-μL hexane plugs injected into a GC-FID did not give appreciably different chromatograms regardless of whether Optima hexane or ACS-grade hexane (BDH, delivered in glass bottles) was used for rinsing or for injection. Subsequently, ACS-grade hexane was used for rinses, and Optima for dilutions. Newly-received bottles of ACS-grade hexane were checked for possible impurities by GC before use. Water used for rinsing was distilled, de-ionized and filtered (0.2 μm) (Nanopure II system, Barnstead-Thermolyne, Dubuque, IA). Additionally, water used to prepare solutions was extracted 3 times with Omnisolv-grade chloroform to ensure a minimum of lipid contaminants. This extraction is consistent with the treatment of water used to prepare lipid extracts from marine material (see Parrish, 1998a).

Nine individual neat sterol standards, plus a coprostanone (5β-cholestan-3-one) standard, were used for sterol identification. The following standard mixtures were prepared in hexane or 1:1 hexane/chloroform: Mixture 1 (*epi*coprostanol, coprostanone, cholesterol,

cholestanol, stigmasterol), 85 μg/mL total sterols; Mixture 2 (coprostanol, campesterol, lanosterol, sitosterol, fucosterol), 57 μg/mL total sterols. Lanosterol (~65%), fucosterol and coprostanol were obtained from Steraloids (Wilton, NH), while all other sterols, coprostanone and cholestane (internal standard) were purchased from Sigma (St. Louis, MO).

Samples were analyzed using a Varian 3400 gas chromatograph with a flame ionization detector. GC-MS analysis was done on a Hewlett-Packard 5890 II GC with a 5971A Mass Selective Detector using 70 eV electron ionization.

2.2 Sampling

Sediment cores were obtained from an off-shore site in Trinity Bay (St-7) (Fig. 1.6) and two in-shore sites in the Northwest Arm of Trinity Bay (H-1, H-9) with a 30-cm box corer during the spring and summer of 1994. Moored sediment trap arrays (Parrish, 1998b) were deployed at St-9 and St-7 during 1994 and 1995 at depths of 50, 75 and 100 m, with four collectors at each depth. Finally, vertical (100 m depth to surface) and horizontal plankton net tows (20 μm mesh) (the latter at the depth of maximum chlorophyll fluorescence, as determined with a Seabird CTD equipped with a SeaTech fluorimeter) were taken at sites St-7, St-9, St-11 during March, April, May and June of 1996, at H-9 in March and April 1996, and along a transect (T-1, 2, 3, 4) leading into the harbour of the town of Trinity during April 1995. Sample collection and storage are described in detail in Parrish (1998b).

2.3 Sample preparation

An overview of the extraction and analysis methods used is shown in Fig. 2.1. Sediments were processed as described by Favaro (1998). Cores were sectioned into 2-cm portions (depth) after removal of the outer 1 cm (diameter) of each core. Sections were air-dried overnight in low light conditions and visible debris removed. Sediments were ground with a mortar and pestle and ~ 10 g of each section, plus ~ 1 g pre-cleaned anhydrous Na_2SO_4 was Soxhlet extracted for 24 hours with 9:1 dichloromethane:methanol in a pre-extracted cellulose thimble. One quarter of the extract, previously evaporated to dryness and stored frozen, was re-dissolved in 1 mL of 2:1 (v/v) hexane:chloroform by sonication (ice bath, 1 minute) for analysis.

Sediment trap and net tow samples were collected on pre-combusted GF/C glass fibre filters, extracted with 2:1 chloroform:methanol by a modified Folch procedure (Folch *et al.*, 1959; Parrish, 1998a), and the lipids recovered in chloroform to give a total lipid extract (TLE). Since there was often a considerable length of time (up to several months) between extraction and analysis, all TLEs were stored in the dark under N_2 at -20 °C until analysis.

2.4 Saponification, derivatization and analysis

For sterol analysis, 0.1 or 0.5 mL of sample extract (depending on availability and anticipated sterol concentration), was evaporated to dryness under a gentle flow of nitrogen (UHP or HP+ grade), then saponified with 0.1 mL saturated (12% w/v) KOH in methanol (75 °C, 1 h). After the addition of water (0.4 mL), the solution was shaken and extracted with

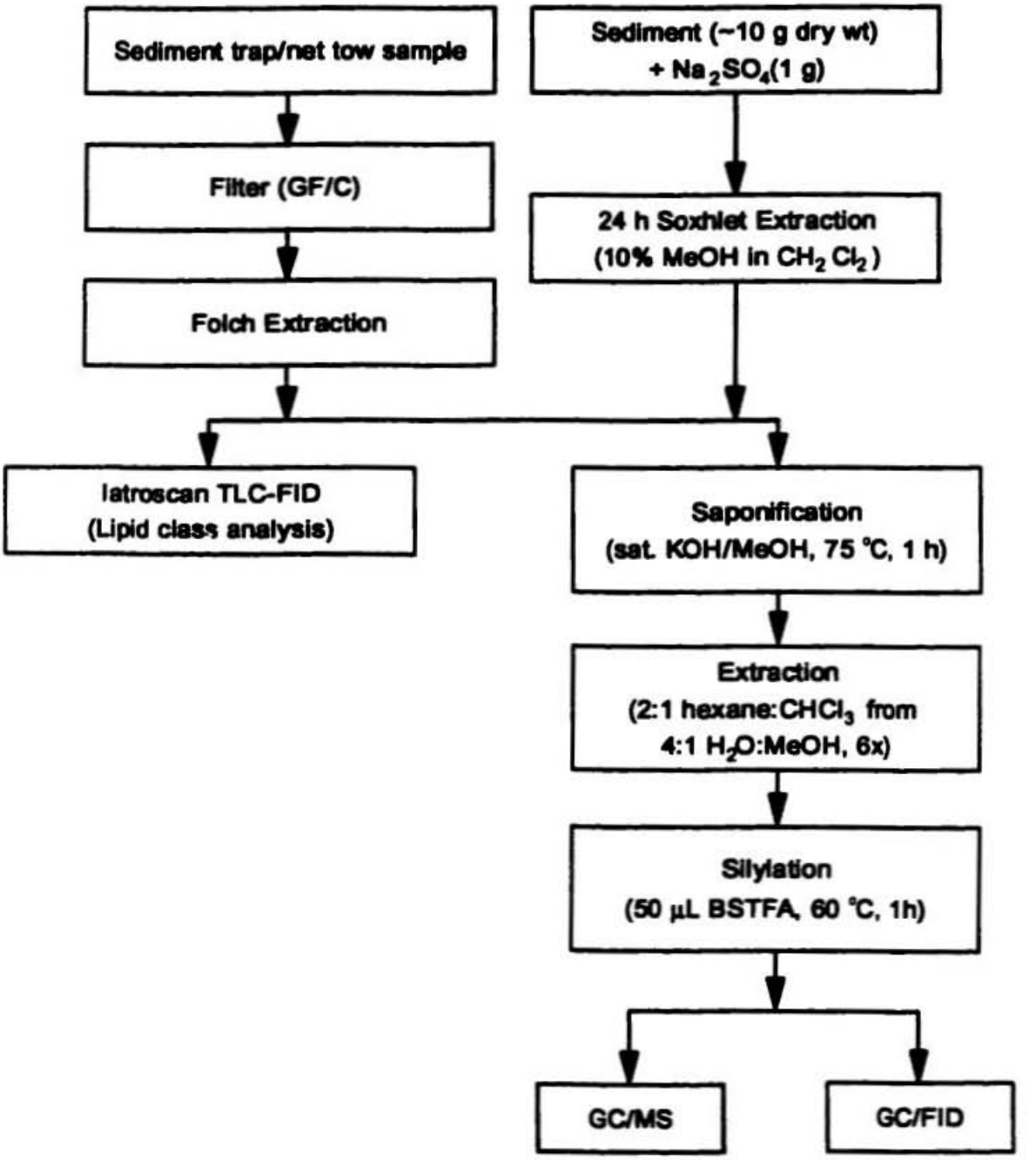


Figure 2.1. Summary of analytical procedures used for sterols

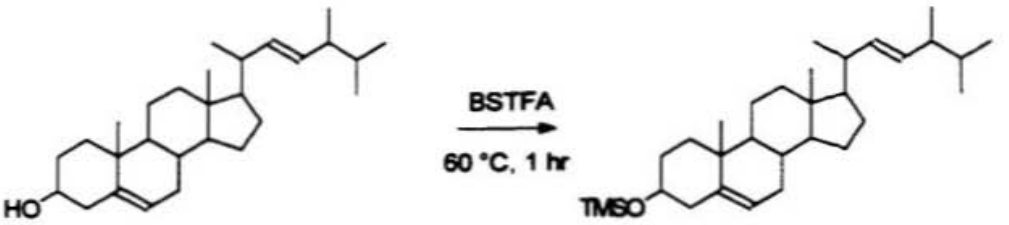


Figure 2.2. Sterol derivatization

6 aliquots (0.1-0.2 mL each) of 2:1 hexane:chloroform. To assist phase separation, a drop of saturated NaCl solution was added or the sample was centrifuged. The pooled extracts were evaporated to dryness and derivatized with 50-200 μL of bis-*N,O*-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Supelco, Bellefonte, PA) (60 °C, 1 h) to produce the trimethylsilyl (TMS) ethers (Fig. 2.2). Where necessary, 6 drops of silylation-grade pyridine (Supelco, Bellefonte, PA) were added to dissolve all the material before derivatization. Any samples not chromatographed immediately after derivatization were stored in a desiccator at -20 °C with the derivatizing agent still in place. However, in accordance with the observations of Jones *et al.* (1994) concerning storage of sterol TMS ethers, samples were generally analyzed within 24 hours of derivatization.

Immediately prior to analysis, the derivatizing agent was evaporated under N_2. The TMS ethers were dissolved in 10-25 μL hexane containing cholestane as an internal standard (48 μg/mL or later, 90 μg/mL) and analyzed by capillary GC-FID on a 30 m x 0.25 mm I.D. (5% phenyl)polydimethylsiloxane column (DB-5, 0.12 µm film; J & W Scientific, Palo Alto,

CA). Cholestane is a widely-used internal standard in sterol analysis (e.g., Smith *et al.* 1983; Nichols and Espey, 1991; Quemeneur and Marty, 1992). Steranes had been previously confirmed as being absent from the TLEs (Favaro, 1998).

Table 2.1. Gas Chromatographic conditions used for sterol TMS ethers.

	GC-FID	GC-MS
Injector port temperature	290 °C	290 °C
Detector/transfer port temperature	315 °C	220 °C
Oven temperature program	80 °C for 1 min., 50 °C/min to 235 °C, 5 °C/min to 305 °C, 305 °C for 15 min.	80 °C for 1 min., 50 °C/min to 200 °C, 5 °C/min to 300 °C, 305 °C for 5 min.
Carrier gas pressure/flow	20 psi constant pressure	1 mL/min constant flow

Each sample was also analyzed by GC-MS on a 25 m x 0.25 mm I.D. CP-Sil 5CB column (100% polydimethylsiloxane; 0.12 μm film; DB-1 equivalent; Chrompack, the Netherlands). Table 2.1 indicates GC conditions for GC-FID and GC-MS analysis; helium was the carrier gas in both cases. For GC-MS, the mass selective detector was scanned from 35-530 amu at 1.5 scans/second. Sterol TMS ethers were identified by their mass spectra and retention times compared with standards and published spectral data (Goad, 1991; Jones *et al.*, 1994) (Table 2.2). Peak areas from GC-FID chromatograms (Varian Star 4.5 integration software) were used for quantitation except where otherwise stated.

2.5 Analysis of total free sterols

Total free sterols in all TLEs were determined by TLC-FID on Chromarods S-III (Parrish, 1987) using an Iatroscan Mk V (Iatron Laboratories, Tokyo, Japan). The development procedure is discussed in more detail in Section 4.4. Each extract was simultaneously analyzed in triplicate on adjacent Chromarods. Cholesterol was used as an external quantitation standard and hexadecan-3-one as an internal standard.

2.6 Floristic analysis and core dating

Subsamples of the plankton tow and sediment trap samples were taken prior to filtration and preserved with Lugol's iodine and 10% buffered aqueous formaldehyde (1 mL each). A 200-μL aliquot was observed at 200 x magnification under an inverted Zeiss compound microscope. An ocular micrometer and appropriate geometric shapes were used to calculate biovolumes (Budge and Parrish, 1998).

Subsamples from three sediment cores (St-7, H-1, H-9) were dated using ^{210}Pb by Dr. Jack Cornett at Mycore Inc., Deep River, ON. ^{210}Pb ($t_{½}$ = 22 years) is a decay product of atmospheric ^{222}Rn which is deposited from the atmosphere onto the ocean surface at a stable rate and incorporated into settling particles and thus into sediments (Schell, 1982).

Table 2.2. Elution order and characteristic GC-MS ions for sterols detected. No single sample contained all the sterols described.

Trivial name	*Abbreviation*	*Characteristic ions*
24-nordehydrocholesterol	24nor-26$\Delta^{5,22E}$	442, 255, 313, 129, 97
24-nordehydrocholestanol	24nor-26Δ^{22E}	444, 374, 345, 257
coprostanol	5β-27Δ^{0}	445, 370, 316
*epi*coprostanol	3α,5β-27Δ^{0}	445, 370, 316
occelasterol	24Me-24nor-27$\Delta^{5,22E}$	456, 366, 255, 111
trans-22-dehydrocholesterol	27$\Delta^{5,22E}$	456, 366, 255, 111
trans-22-dehydrocholestanol	27Δ^{22E}	374, 257
cholesterol	27Δ^{5}	458, 443, 368, 353, 129
cholestanol	27Δ^{0}	460, 445, 370
desmosterol	27$\Delta^{5,24}$	456, 372, 343, 129
brassicasterol	24Me-28$\Delta^{5,22E}$	470, 455, 380, 341, 129
brassicastanol	24Me-28Δ^{22E}	472, 374, 345
24-methylenecholesterol	24Me-28$\Delta^{5,24(28)}$	470, 386, 374, 129
24-methylenecholestanol	24Me-28$\Delta^{24(28)}$	472, 255, 215
24-methylcholesterol	24Me-28Δ^{5}	472, 382, 129
4-methylcholestanol	4αMe-28Δ^{0}	474, 459, 384, 229
24-methylcholestanol	24Me-28Δ^{0}	474, 459, 384, 215
ethylcoprostanol	24Et-5β-29Δ^{0}	473, 398, 257, 215
dimethyldehydrocholestanol	4α,24diMe-29Δ^{22E}	486, 388, 357
ethylcholesta-5,22*E*-dienol	24Et-29$\Delta^{5,22E}$	484, 394, 255, 129
ethylcholest-22*E*-enol	24Et-29Δ^{22E}	486, 374, 345, 257
ethylcholesterol	24Et-29Δ^{5}	486, 396, 129
ethylcholestanol	24Et-29Δ^{0}	488, 398, 215
fucosterol	24Et-29$\Delta^{5,24(28)E}$	484, 386, 296, 129

Table 2.2. (Continued)

fucostanol	24Et-29$\Delta^{24(28)E}$	486, 388, 373, 345
dehydrodinosterol	4α,23,24triMe-30$\Delta^{5,22E}$	388, 359, 297, 129, 69
isofucosterol	24Et-29$\Delta^{5,24(28)Z}$	484, 386, 296, 129
isofucostanol	24Et-29$\Delta^{24(28)Z}$	486, 388, 373, 345
dinosterol	4α,23,24triMe-30Δ^{22E}	500, 388, 359
unidentified C_{30},Δ^5 steratrienol	30$\Delta^{5,x,x}$	496, 313, 129

3. Results and discussion

3.1 Method validation

3.1.1 Completeness of derivatization

Conversion of sterol standards to their TMS ethers (Section 2.4) was generally very efficient, with residual free sterols detected in only one instance in the examination of a GC/MS extracted ion chromatogram. These free coprostanol, cholesterol and stigmasterol peaks had areas of 3.85%, 3.66% and 10.6%, respectively, of the peaks for their TMS ether derivatives (quantitation by GC/MS peak areas, assuming equal detector responses), although the first is an underestimate due to the contribution of coprostanone to the coprostanol TMS ether peak. The temperature and reaction time used are similar to those found satisfactory by many workers (e.g., Bayona *et al.*, 1989; Grimalt *et al.*, 1991; Laureillard and Saliot, 1993; Jones *et al.*, 1994; Nichols *et al.*, 1996), although Bull *et al.* (1998) allowed 12 hours for 5β-stanols from soils to react completely with BSTFA. No rearrangements of the standard sterols occurred during the saponification step, which each one being detected both before and after the procedure. Lastly, it was assumed that derivatization efficiency differed negligibly between different sterols, since the unavailability of a wide range of standards would in any case not allow this to be tested for all the sterols detected in the samples.

3.1.2 Linearity and equivalency of detector response

Since Jones *et al.* (1994) caution that FID response for different sterol structures may

differ up to two-fold, the FID responses of different sterols were studied to determine if correction factors would be required. Three-component standards (cholesterol, coprostanol and stigmasterol) were prepared in hexane at 3 different concentrations; serial dilutions from these gave a further four concentrations. These three sterols were chosen to represent a variety of structural features (C_{27}-5β-stanol, C_{27}-Δ^5-sterol, C_{29} steradienol). A 100-μL aliquot of each standard was evaporated to dryness, derivatized with 50 μL BSTFA (Section 2.4), and made up to 25 μL with hexane containing 0.0899 mg/mL cholestane. Each was analyzed in triplicate by GC-FID. Calibration curves, with 95% confidence intervals around the regression lines, are shown Appendix 1. The following linear fits were obtained, with x and y representing sterol concentration (mg/mL) and (sterol FID response/ internal standard FID response) (unitless), respectively:

Coprostanol:	$y = 105\ (\pm 6)\ x\ -\ 0.0\ (\pm 0.3)$,	$r^2 = 0.946$ (n=21)
Cholesterol:	$y = 106\ (\pm 4)\ x\ -\ 0.8\ (\pm 0.8)$,	$r^2 = 0.978$ (n=21)
Stigmasterol:	$y = 107\ (\pm 13)\ x\ -\ 0.9\ (\pm 1.1)$,	$r^2 = 0.767$ (n=21)

While the correlation coefficient for the stigmasterol calibration curve was poorer (r^2 = 0.767), all three correlations were significant ($P<0.01$) (Sokal and Rohlf, 1981). Furthermore, the standard deviations for the line slopes all overlap, suggesting that detector response is not significantly different for these three sterols, and the standard deviations of

the y-intercepts all overlapped zero. Given this, and given that the paucity of standards available would preclude the calculation of FID response correction factors for all the sterols detected, all subsequent data was processed assuming equal detector response for all sterols.

3.1.3 Recovery of free sterols and steryl esters

Standard solutions of free cholesterol and of cholesteryl hexadecanoate (palmitate) were prepared in hexane, and aliquots of each dispensed in order to deliver 3.8 μg of cholesterol or its equivalent. Two free cholesterol and two cholesteryl palmitate samples were saponified, extracted and derivatized as per Section 2.4.

Table 3.1 Detector responses for equivalent amounts of cholesterol in free and esterified form

Form of cholesterol	Cholesterol FID response/Internal standard FID response (mean ± SD., n=2)
Free cholesterol	1.94 ± 0.03
Cholesteryl palmitate	1.9 ± 0.2

The amount of cholesterol detected after saponification and derivatization did not differ significantly (two-tailed Student's t-test, $P<0.05$) between cholesteryl palmitate and free cholesterol (Table 3.1). This result suggests that steryl esters are readily detected by the method, although only one compound from each class was used.

3.1.4 Effectiveness of the liquid/liquid extraction step

To gauge whether sterols are completely extracted from the aqueous layer into the organic phase following saponification, an aliquot of saponified sediment material (St-7, 2-4 cm cut) was extracted 6 times with hexane/chloroform as described (Section 2.4). The aqueous layer was then re-extracted with a further 6 aliquots of hexane/chloroform, and these extracts pooled separately. Both lots of extracts were separately derivatized and analyzed for sterol TMS ethers.

The few peaks in the sterol region of the chromatogram of the second set of extractions are an order of magnitude smaller that the same peaks from the first extractions (Fig. 3.1). Furthermore, several do not appear to correspond to peaks in the first set of extractions, suggesting that they are contaminants rather than remaining analytes. These results suggest that sterol extraction during this step is very efficient.

3.2 Plankton composition of the samples

Abundances of different phytoplankton taxa in the net tows and sediment traps are reported in detail by Budge and Parrish (1998). From March to May 1995, centric diatoms are the most abundant algae in net tows. *Chaetoceros* and *Thalassiosira* (Centrales) each constitute 11% of algal cells in March, with unidentified centric diatoms comprising a further 50% and dinoflagellates (particularly *Ceratium tripos*, 14%) contributing nearly 20%. The centric diatoms *Chaetoceros* (24%) and *Leptocylindrus danicus* (48%) constitute the bulk

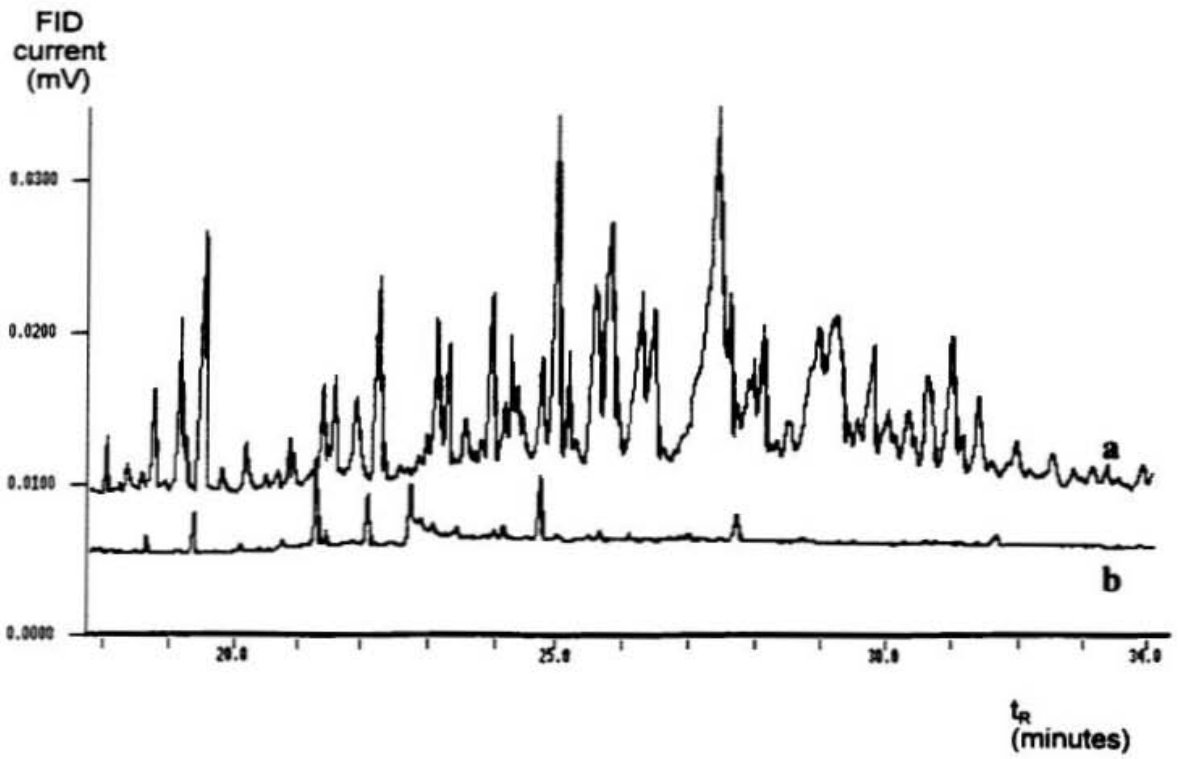


Figure 3.1. GC-FID chromatograms of two consecutive sets of extractions of sediment TLE saponification mixture (St-7, 2-4 cm). a. First 6 extractions. b. Next 6 extractions. 1 μL of 25 μL injected each time.

of the April net tow, with *Chaetoceros* becoming even more important in May (91%). The June net tow is unique for the large dinoflagellate contribution from *C. tripos* (85%). Copepod nauplii were also present in many net tow samples (Parrish, 1998b).

Diatoms predominated in the sediment traps. In the near-shore (St-9) traps recovered in July *Leptocylindrus* (Centrales), *Grammatophora* (Pennales) and unidentified centric diatoms each constituted 33% of algal cells. In the off-shore (St-9) trap recovered in April 1995 after an overwinter deployment, *Coscinodiscus* (Centrales) and *Fragillaria* (Pennales) contributed 73% and 22% of algal cells, respectively.

3.3 Sterols in Trinity Bay samples

3.3.1 Reported approaches to using sterol biomarkers

Sterols have been used in a variety of ways to assign and distinguish sources of organic matter. A straightforward approach is the assignment of each specific sterol to a particular organism or source. Thus, Colombo *et al.* (1997) detected 14 sterols in sediments in the Laurentian Trough and grouped them into 4 categories (phytoplankton, zooplankton, and terrestrial plants sources, with coprostanol reported separately). However, as previously noted, a specific sterol may be synthesized by several widely different taxa (Volkman, 1986), and therefore this approach is problematic if there is no supporting evidence for the assignment of a particular sterol to a particular source taxon. Before such source assignment can be used to establish the origin of organic matter in the marine environment, any supporting evidence (other biomarkers, algal remains) must also be considered.

Huang and Meinschein (1979) first proposed the ratio of C_{27} to C_{29} sterols as an ecological source indicator, although the simple assignment of sterols to a specific source based only on carbon number is problematic (Section 1.4.1; see also Volkman, 1986). Used with caution, parameters such as the total of C_{28} and C_{29} sterols , and the ratio $27\Delta^5/(29\Delta^{5,22} + 29\Delta^5)$ (Li *et al.*, 1995) can still serve as indices of terrestrial input. Such parameters are best used to establish source or preservation trends within a series of samples (e.g., from different locations along a transect), rather than as absolute indicator of source material.

Δ^5-Stenols are more suspectible to biodegradation in oxic waters than are sterols with saturated steroid nuclei (stanols) (Volkman *et al.*, 1981). Furthermore, the stanols may be formed by the biohydrogenation in organic matter (Volkman *et al.*, 1981; Grimalt *et al.*, 1991), although certain microalgae (Patterson, 1991) and some marine invertebates (e.g., sponges, echinoderms, jellyfish, tunicates: Volkman *et al.*, 1981) synthesize stanols. Thus, the ratio of stanols to their Δ^5-unsaturated analogues ("stanol/stenol ratio") is a useful indicator of the preservational status of organic matter (e.g., Li *et al.*, 1995). In marine sediments, stanols will generally be present along with their stenol analogue (Cranwell, 1982). For sedimentary sterols, this ratio is dependent on the redox potential and depositional environment. Furthermore, the variation in stanol/stenol ratio with different carbon numbers may suggest preferential degradation of material from certain sources (Cranwell, 1982). For example, terrestrial sterols may be less susceptible than marine sterols to degradation in the marine environment due to being associated with relatively refractory humic material.

Finally, multivariate statistical techniques such as principal component analysis

(Mazaud *et al.*, 1989; Colombo *et al.*, 1996; Mudge and Norris, 1997) have been used to establish co-variation between individual sterols and between sterols and other biomarkers, thereby increasing the certainty of source assignment and thus the origin of the material. Linear correlation coefficients between different sterols have also been used to group them according to source (Bayona *et al.*, 1989).

In the following discussion, the sterol composition of Trinity Bay plankton tow samples, settling particles and sediments will be considered in the light of supporting evidence from floristic analysis, other biomarkers and probable inputs based on sampling location. The individual sterol species thus having been attributed, the contributions of their sources (phytoplankton, zooplankton, higher plants, macroalgae) to organic matter will be elucidated.

3.3.2 Plankton

The spring diatom increase (spring bloom) is the major annual primary production event in Newfoundland coastal waters (Parrish, 1998b). At this time, primary productivity (g C fixed/m^2/day) is equivalent to that in highly productive upwelling regions.

Plankton samples contained 0.4 ± 0.4 μg/g dry weight total free sterols (mean ± standard deviation, n=13) during the spring of 1995 (April, May), ranging across an order of magnitude (158-1542 μg/g dw), and 1.4 ± 1.3 μg/g dw (n=26) during the spring of 1996 (March-June), suggesting considerable annual variation. During 1996, there is a clear increase in sterol content of the material from March to June across all stations (Fig. 3.2), with a corresponding increase in percent sterol content, although this peaks in May.

Furthermore, in all months sterol content in the horizontal tows decreases from off-shore to near-shore sites (Fig. 3.3), although station H-9, located far into the Northwest arm from the open Trinity Bay, deviates from this in all cases, possibly due to the additional contribution of sterols from terrestrial material. Of the types of samples, plankton tows generally had the simplest sterol composition, with 3 to 14 sterols being identified (Fig. 3.4). Figure 3.5 shows the mean composition of the plankton. Not all sterols were detected in all samples. In most cases, a few sterols made up the bulk of the total. C_{27} sterols predominated, with desmosterol (mean 26%; range 12 - 47%), cholesterol (24% , 7 - 8%), and *trans*-22-dehydrocholesterol (13%, 8 - 9%) consistently present. In many samples, the C_{26} sterol 24-nordehydrocholesterol was also prominent, as was a sterol which is likely occelasterol (Harvey and Johnston, 1995). (While *cis*-22-dehydrocholesterol has an identical mass spectrum and similar elution behaviour (Jones *et al.*, 1994) to this sterol, *cis*-22-sterols are rare in marine samples.) In some samples (St-11, H-9, all tows from St-7 and St-9), 28-methylenecholesterol was also prominent (5.5- 18.7% of total sterols), and the corresponding 28-methylenecholestanol was also present in most of these cases (3.8- 10.3% of total). Some C_{29} sterols were found in certain samples, and these consisted mostly of fucosterol and isofucosterol and their corresponding 5α-stanols. No C_{30} sterols were detected.

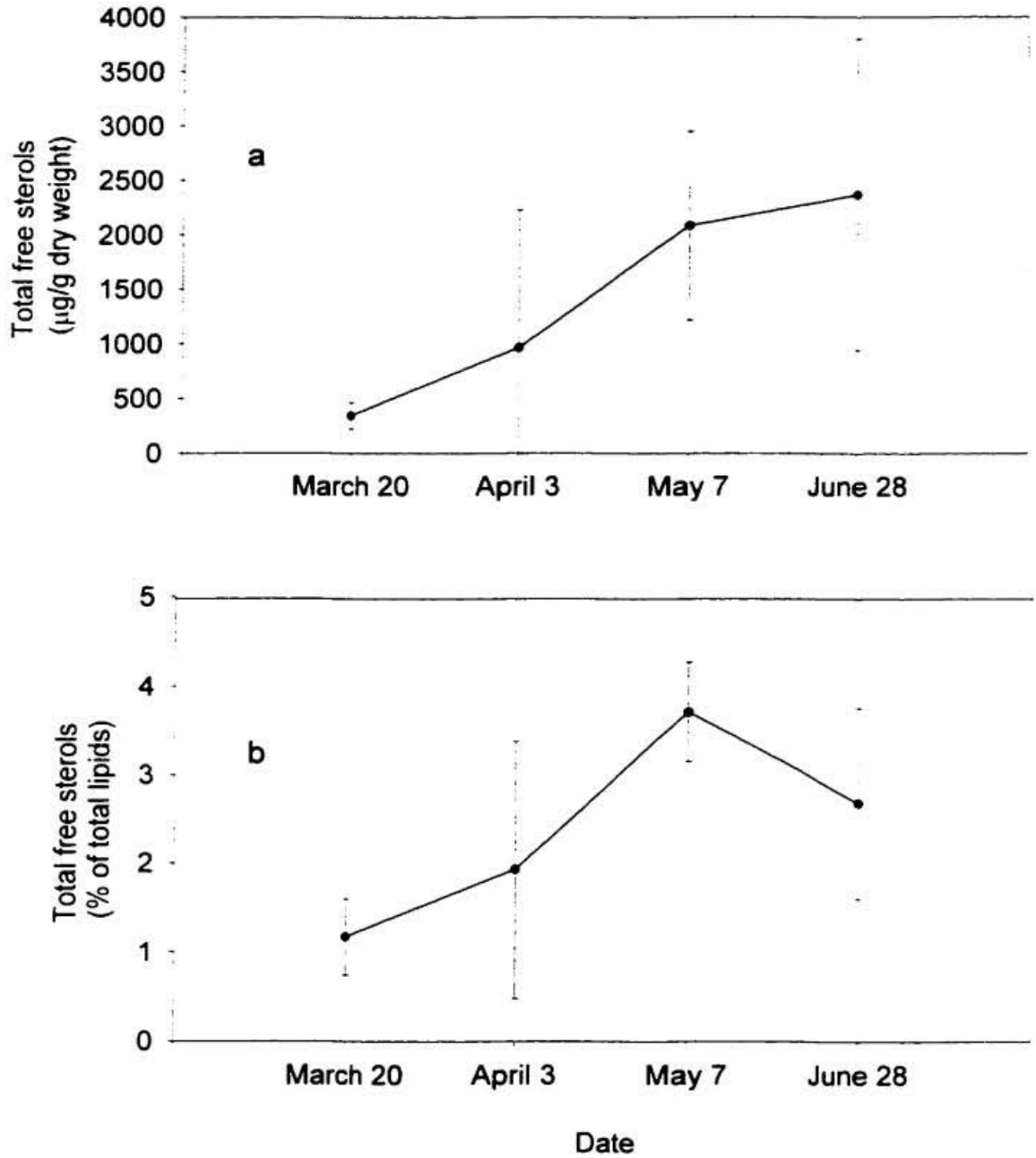


Figure 3.2. Seasonal variation in plankton sterol content during 1995. St-7, St-9, St-11; mean ± SD, n=6-7.

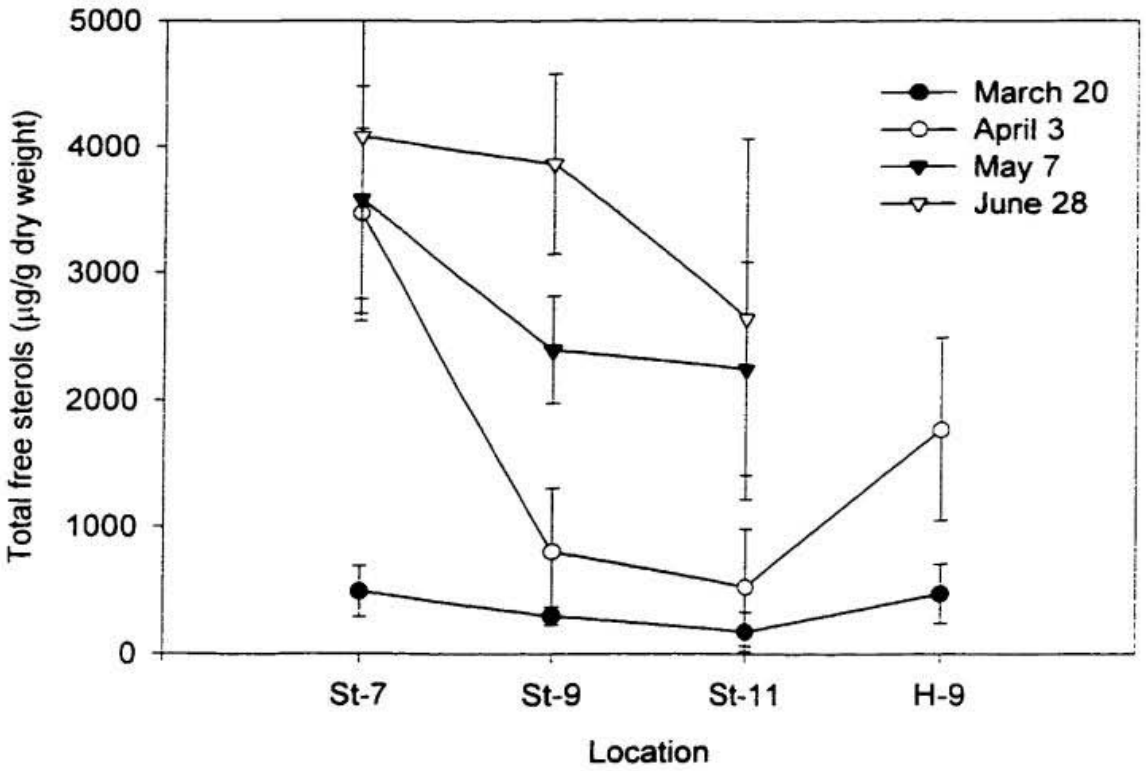


Figure 3.3. Spatial variation in plankton sterol content during 1995. Mean ± SD. n=3.

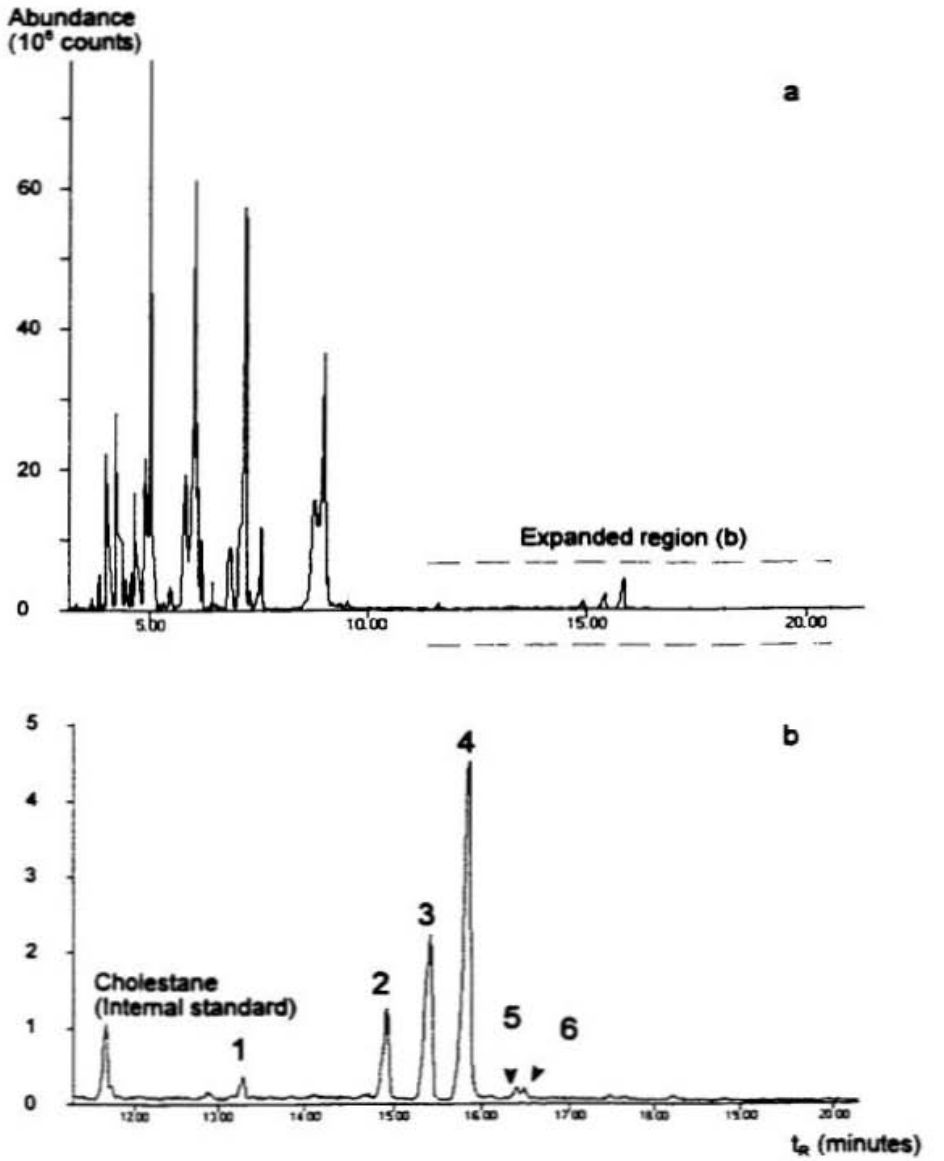


Figure 3.4. a. GC/MS total ion chromatogram for the H-9 horizontal plankton tow sample (April 1995). b. sterol region. Peaks: 1. 24nor-26$\Delta^{5,22E}$; 2. 27$\Delta^{5,22E}$; 3. 27Δ^{5}; 4. 27$\Delta^{5,24}$; 5. 24Me-28$\Delta^{5,24(28)}$; 6. 24Me-28$\Delta^{24(28)}$. Abbreviations are those given in Tables 1.1 and 2.2.

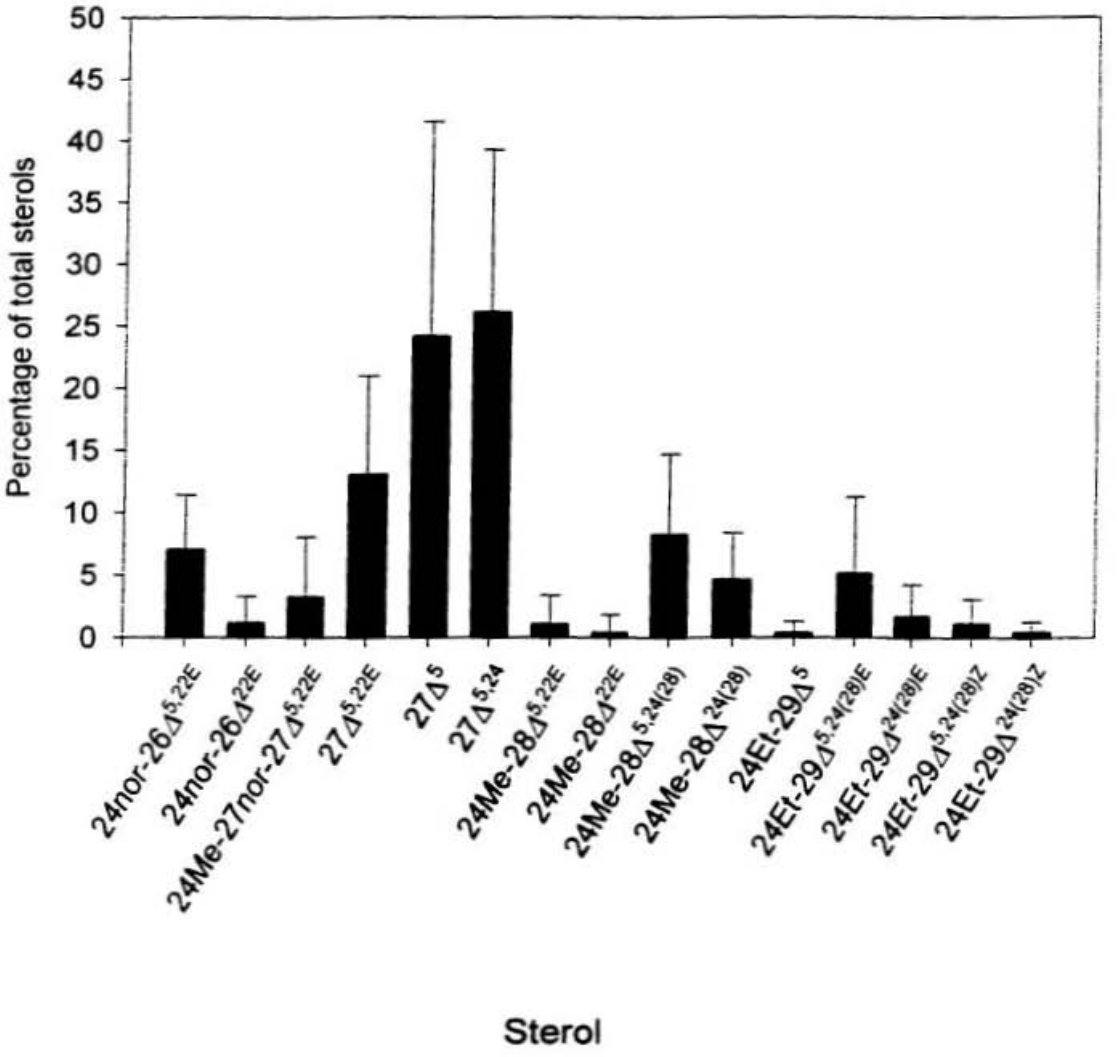


Figure 3.5. Mean sterol composition of Trinity Bay plankton tows. Mean ± SD; n = 10. Both vertical and horizontal tows are included

These compositions are fully consistent with a marine origin with major contributions from diatoms. Desmosterol is the major sterol of several *Chaetoceros* species (Gladu, 1989), and cholesterol is an important component of others (Patterson, 1991). The C_{28} sterols detected, brassicasterol and 24-methylenecholesterol, are among those often associated with diatoms (Volkman, 1986; Patterson, 1991). Particularly, 24-methylenecholesterol is the major sterol of *Thallasiosira* (Volkman and Hallegraeff, 1988). While fucosterol and isofucosterol are normally associated with macrophytic brown algae (Patterson, 1991), they may also suggest that zooplankton present are rapidly dealkylating any ethylcholesterol present, since fucosterol and isofucosterol are intermediates in this process (Serrazanetti *et al.*, 1989). Higher plants (terrestrial plants, seagrasses) may be the source of any C_{29} sterols being dealkylated in the plankton samples, but the fatty acid composition of the samples is inconsistent with higher plant input (Budge and Parrish, 1998). A last possible source of fucosterol is *Chaetoceros*, in which this sterol has been detected (<17%) (Gladu, 1989). The *trans*-22-dehydrocholesterol present is likely due to zooplankton (Serrazanetti *et al.*, 1989), as are the C_{26} (24-nor) sterols (Volkman *et al.*, 1981). The absence of C_{30} sterols reflects that the most important dinoflagellate contributions were in June, while all the plankton samples considered were taken earlier in the year.

Stanol/stenol ratios were generally low, with the corresponding stanols of the major sterols cholesterol and *trans*-22-dehydrocholesterol being entirely absent. This suggests little bacterial degradation of the material, an observation supported by low hydrolysis/lipolysis indices (Parrish, 1998b).

The plankton illustrate one of the weaknesses of comparisons based on percent sterol composition, namely that the value for each sterol may influence those for all others in the same sample. For example, it is possible that sterols besides desmosterol (32%) and cholesterol (68%) were present in the T-1 plankton tow, but since they were below the detection limit, the percentage contributions of the two sterols which could be quantified appear inflated. Nonetheless, these undoubtedly remain the major sterols.

3.3.3 Settling particles

Settling marine particles transport biogenic carbon, and pollutants, from surface waters where primary production occurs to the benthic community and to marine sediments (Colombo *et al.*, 1996). This connection between pelagic and benthic food webs has implications for commercial fisheries. Their additional importance in the context of global climate regulation have already been discussed.

Settling particles collected at St-7 at 50, 75, and 100 m depths in April 1995 contained 0.4 ± 0.2 mg/g dw total free sterols (mean ± SD, n=7). While the availability of only a single datum at 75 m precluded analysis of variance on all three depths, the concentrations did not differ significantly between the 50 and 100 m arrays ($P>0.05$), suggesting that sterols are not lost by conversion to non-sterol molecular species during sinking through the upper water column.

Cholesterol ($17 \pm 5\%$, n=6), desmosterol ($24 \pm 6\%$) and *trans*-22-dehydrocholesterol ($10 \pm 2\%$) and brassicastanol ($9 \pm 6\%$) were among the principal sterols, clearly reflecting the

plankton source of the material; however, unlike in the plankton, appreciable proportions of 24-methylcholesterol (6 ± 2%) and 24-ethylcholesterol (5 ± 3%) were also found. Furthermore, stanol/stenol ratios were generally higher than for plankton samples from this site, suggesting some biodegradation in the water column.

The number of individual sterol species detected increased with depth (Fig. 3.6), particularly between 75 m and 100 m. 24-Ethylcholestadienol and the corresponding stanol 24-ethylcholest-22-enol were only detected at 100 m depth. Stanol/stenol ratios for 24-nordehydrocholestanol/24-nordehydrocholesterol, *trans*-22-dehydrocholestanol/*trans*-22-dehydrocholesterol, and 24-ethylcholestanol/24-ethylcholesterol were at a maximum at 100 m, although the cholesterol/cholestanol ratio was at a minimum. Furthermore, the proportion of fucosterol decreases with depth. None was detected at 100 m, and its transformation to 24-ethylcholesta-5,22-dienol during the C-24 dealkylation process by zooplankton may explain this (Serrazanetti *et al.*, 1989).

Settling particles intercepted at the same depths at the near-shore site (St-9) in June 1994 contained many of the same sterols as the plankton samples, with cholesterol (19 ± 8%, n=3) most common, and brassicasterol more abundant (9 ± 3%) than in the plankton. In contrast to the St-7 samples, 24-ethylcholesterol and 24-methylcholesterol and the corresponding stanols are virtually absent. Sterols also comprised a greater proportion of lipids in the settling particles at St-9 in June than at St-7 in April (Table 3.2).

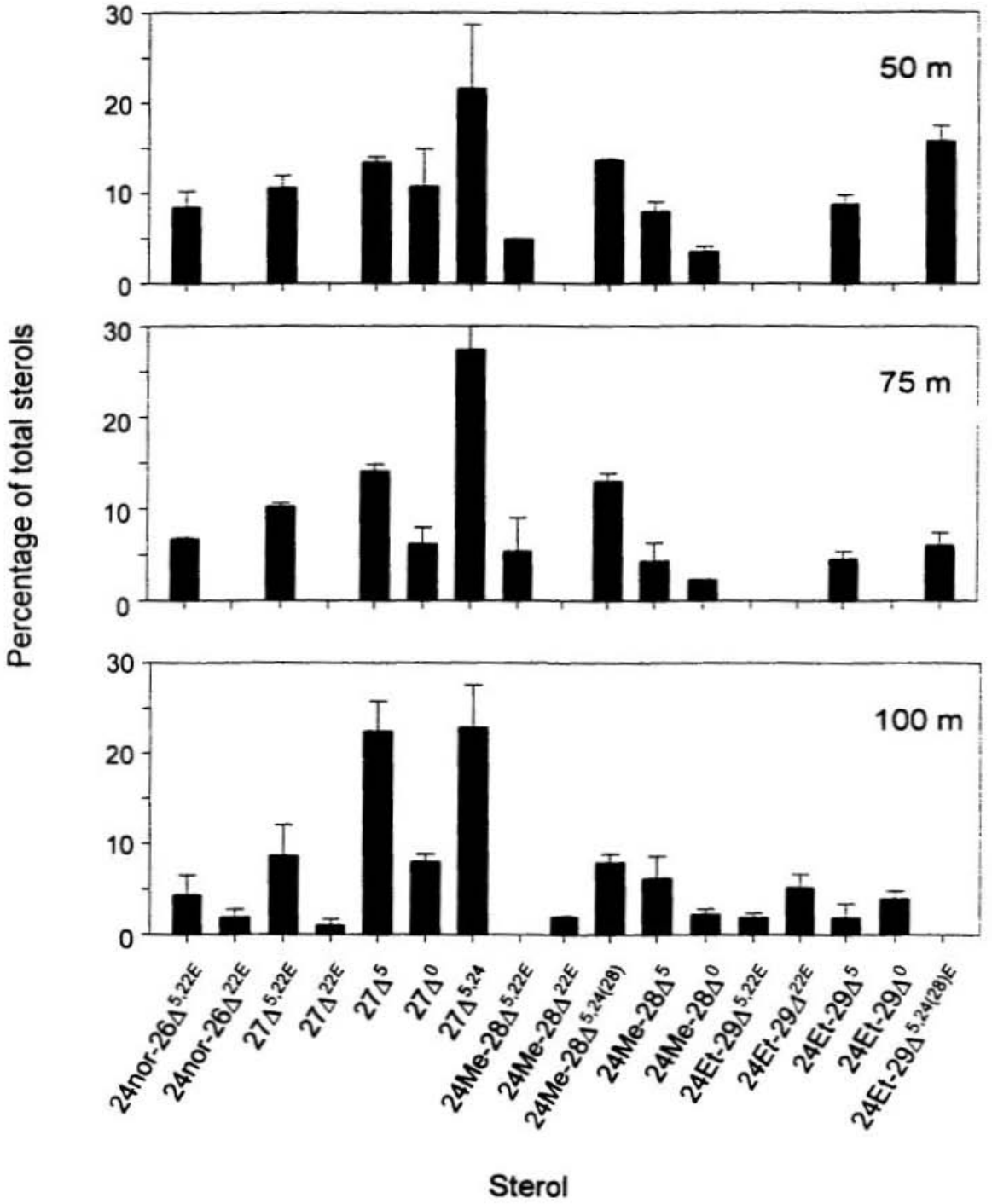


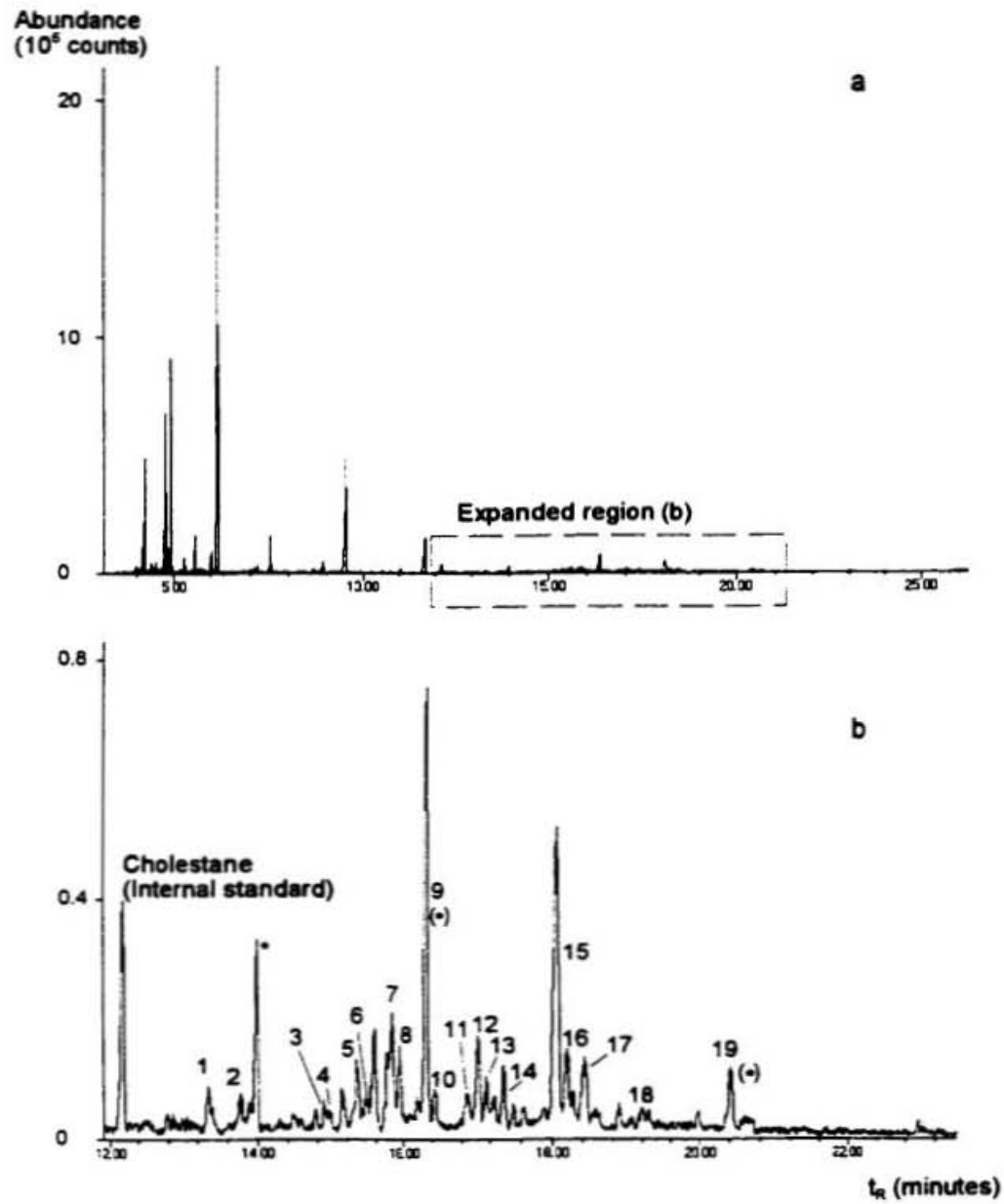
Figure 3.6. Sterol composition of settling particles at St-7. Mean ± SD, n= 2 collectors per depth.

Table 3.2. Free sterol contribution (%) to total lipids in settling particles at near-shore (St-9) and off-shore (St-7) sites (mean ± SD, n = 2-4). Values at all depths differ significantly between sites/seasons ($P<0.05$).

Depth (m)	St-9 (June)	St-7 (April)
50	6.4 ± 0.8	4.6 ± 0.4
75	7.2 ± 0.7	4.6 ± 0.5
100	11 ± 3	3.3 ± 0.6

3.3.4 Sediments

Sediment cores had the most complex sterol compositions (Fig. 3.7), with over 20 sterols being identified in all samples. Several peak identifications are tentative, and a few remain identified only as sterols, based on characteristic fragments (esp. m/z 129 for Δ^5 sterols) (Rahier and Benveniste, 1989). Their retention times suggest C_{28} and/or C_{29} species. Several alkanol TMS ethers (prominent m/z 103 and M-15) (Evershed, 1992), eluted in the sterol region, notably the C_{26}, C_{28}, and C_{32} species, with smaller amounts of C_{30}. Quantitation of brassicasterol was complicated by its co-elution with the C_{28} alkanol in many samples. In processing GC-FID chromatograms, the leading edge of the peak which represented brassicasterol was split off, along with any shoulder present, prior to integration to attempt to account for any contribution from the C_{28} alcohol. Similarly, the C_{32} alcohol co-eluted with a species tentatively identified as a C_{30}, Δ^5 steratrienol ($M^+ = 496$, prominent m/z 129) (Fig. 3.8). However, the M-15 ion often present in sterol mass spectra was not observed. The substance was detected in sediments at H-1 and H-9, but not at the off-shore site St-7, suggesting a terrestrial origin. It may also be a pentacyclic, non-hopanoid triterpenoid


Abundance
(10^5 counts)
a
20
10
0
Expanded region (b)
5.00
10.00
15.00
20.00
25.00
0.8
0.4
0
b
Cholestane
(Internal standard)
1
2
3
4
5
6
7
8
9
(•)
10
11
12
13
14
15
16
17
18
19
(•)
12.00
14.00
16.00
18.00
20.00
22.00
t_R (minutes)

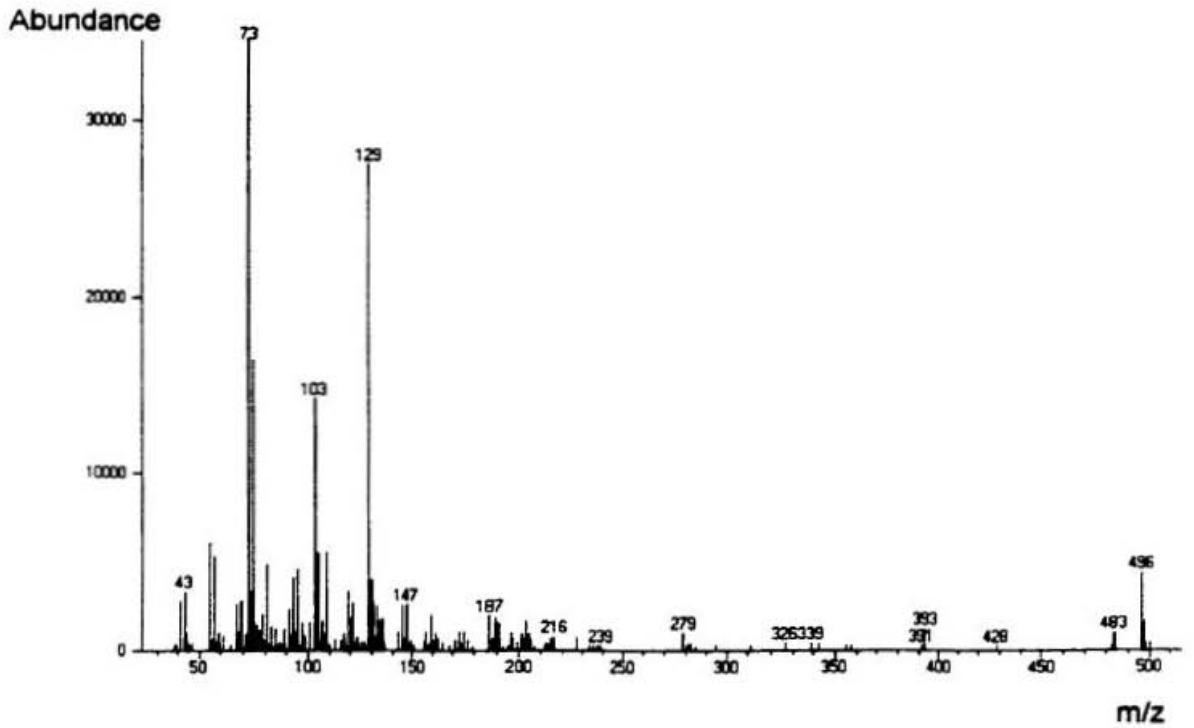


Figure 3.8. Mass spectrum of the peak at t_R=20.45 min in Figure 3.7. The fragment at m/z 103 is due to an alkanol TMS ether co-eluting with the postulated C_{30} steratrienol.

alcohol, such as has been found in terrestrial soils (Bull *et al.*, 1998). Volkman *et al.* (1981) also detected a late-eluting compound with these characteristics in the sterol fraction of lipids from intertidal sediments, but did not comment further on its structure. Further mass spectrometry work is needed to fully elucidate the structure of the substance observed.

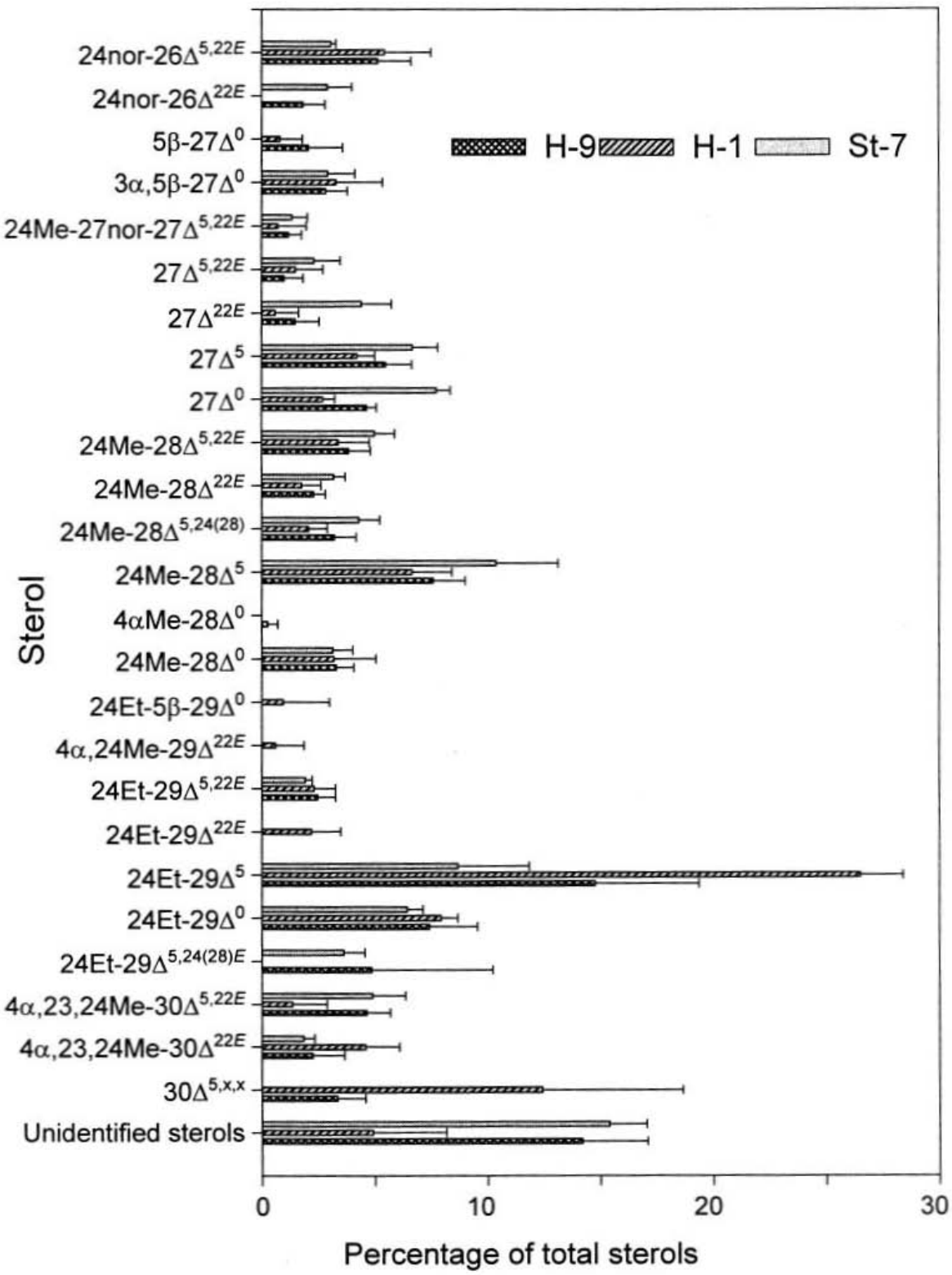
Among the major sterols consistently present are 24-ethylcholesterol, 24-methylcholesterol, brassicasterol, cholesterol, and their stanol analogues, along with the algal sterol 24-methylenecholesterol (Fig. 3.9). The C_{30} 4-methylsterols dinosterol and dehydrodinosterol, suggestive of dinoflagellate input, were also detected at all three sites. Although distinctive dinoflagellate sterols were not detected in the plankton or settling particles, likely due to the dinoflagellate population maximum occurring in summer rather than spring, the presence of these sterols in all sediments cores suggests that dinoflagellate input is well preserved.

All three sites contained substantially higher proportions of C_{29} and C_{28} sterols than the plankton and sediment trap samples, suggesting a greater terrestrial plant contribution. Desmosterol is completely absent from the sediments, despite being a major component of the plankton and sediment trap samples. Zooplankton and bacterial activity may have converted all the desmosterol present to cholesterol prior to burial in the sediments.

3.3.4.1 Spatial variation

Surface sediments contained 31- 129 μg/g dw total free sterols (263- 410 μg/g organic matter) (Table 3.3), comprising 2.6- 7.5 % of total lipids. Sterol concentrations in

Figure 3.9. Sterol composition of Trinity Bay sediments. Whole core means ± SD; n = 8-10 individual horizons.


H-9
H-1
St-7
Sterol
24nor-26Δ5,22E
24nor-26Δ22E
5β-27Δ0
3α,5β-27Δ0
24Me-27nor-27Δ5,22E
27Δ5,22E
27Δ22E
27Δ5
27Δ0
24Me-28Δ5,22E
24Me-28Δ22E
24Me-28Δ5,24(28)
24Me-28Δ5
4αMe-28Δ0
24Me-28Δ0
24Et-5β-29Δ0
4α,24Me-29Δ22E
24Et-29Δ5,22E
24Et-29Δ22E
24Et-29Δ5
24Et-29Δ0
24Et-29Δ5,24(28)E
4α,23,24Me-30Δ5,22E
4α,23,24Me-30Δ22E
30Δ5,x,x
Unidentified sterols
0
10
20
30
Percentage of total sterols

surface sediments (μg/g dw) at H-1 were significantly greater ($P<0.05$) than those at H-9 and St-7, although none of these stations differ when these concentrations are normalized to the organic matter content; the difference in concentrations per gram dw may simply reflect the slower sedimentation rate at H-1 (Favaro, 1998). Mean subsurface (>2 cm depth) sterol concentrations did not differ significantly between stations, ranging from 3.7 - 79 μg/g dw in individual horizons. However, subsurface percentage sterol contributions to total lipids decreased in the order St-7, H-9, H-1 (7.5%, 3.7%, 1.8%, respectively).

The prominence of 24-ethylcholesterol (8 ± 3% of total sterols) and 24-methylcholesterol (10 ± 3 %), at the off-shore site St-7 is noteworthy. It supports other work which indicates a substantial contribution by terrestrial material to sediments at this site (K. Pulchan and C. C. Parrish, *pers. comm.*). These markers, both of which are among the major sterols of higher plants (Goad, 1991), are more abundant than the traditional indicators of

Table 3.3. Total free sterols (mean ± SD) in surface (0-2 cm; n = 2) and subsurface (n = 7) sediments. Values with different superscripts differ significantly between sites (one-way ANOVA, least significant difference test (Miller and Miller, 1988), $P<0.05$). *Means differ significantly (Student's t-test, $P<0.05$) between surface and subsurface.

Location and depth		Total free sterols (μg/g dw)	Total free sterols (μg/g OM)	Free sterols- % of total lipids
St-7	surface	30 ± 5^{*a}	263 ± 46^{a}	7.5 ± 0.3^{a}
	subsurface	21 ± 6^{*a}	245 ± 74^{a}	7.5 ± 1.9^{a}
H-1	surface	129 ± 32^{*b}	410 ± 101^{*a}	2.6 ± 0.7^{b}
	subsurface	46 ± 21^{*a}	167 ± 79^{*a}	1.8 ± 0.8^{b}
H-9	surface	63 ± 3^{*a}	405 ± 17^{*a}	3.7 ± 1.5^{b}
	subsurface	31 ± 14^{*a}	244 ± 105^{*a}	3.7 ± 1.7^{c}

marine algal input (24-methylenecholesterol, brassicasterol) and the marine sterols detected in the traps. The stanol-stenol ratios at this site range from 0.31 to 1.9 (mean 0.95) (Table 3.4). They are highest for typically marine sterols, suggesting that these may be preferentially degraded (Cranwell, 1982; Jasper and Gagosian, 1993).

24-Ethylcholesterol is even more prominent at the in-shore sites than at St-7 (15 ± 5 % and 26 ± 2 % of total sterols at H-9 and H-1, respectively). This is consistent with a terrestrial source for this sterol. Its high proportion at H-1 is easily explained by input of terrestrial matter from the major watershed on Random Island, which drains into Hickman's Harbour. However, 24-methylcholesterol (8 ± 1% at H-9 and 7 ± 2% at H-1) does not increase correspondingly in prominence. This corroborates the contention that it is not as

Table 3.4. Stanol/stenol ratios in Trinity Bay sediments (calculated from whole core mean sterol compositions)

Stanol/stenol pair	*H-1*	*H-9*	*St-7*
24-nordehydrocholestanol/24-nordehydrocholesterol	0.0	0.36	0.95
trans-22-dehydrocholestanol/*trans*-22-dehydrocholesterol	0.40	1.49	1.91
cholestanol/cholesterol	0.64	0.85	1.16
brassicastanol/brassicasterol	0.52	0.59	0.64
24-methylcholestanol/24-methylcholesterol	0.47	0.43	0.30
24-ethylcholestanol/24-ethylcholesterol	0.30	0.50	0.74
Mean	0.54	0.70	0.95

unambiguously a terrestrial sterol as 24-ethylcholesterol, despite its widespread occurrence in terrestrial plants. Stanol/stenol ratios at the in-shore sites are lower (means of 0.55 at H-1

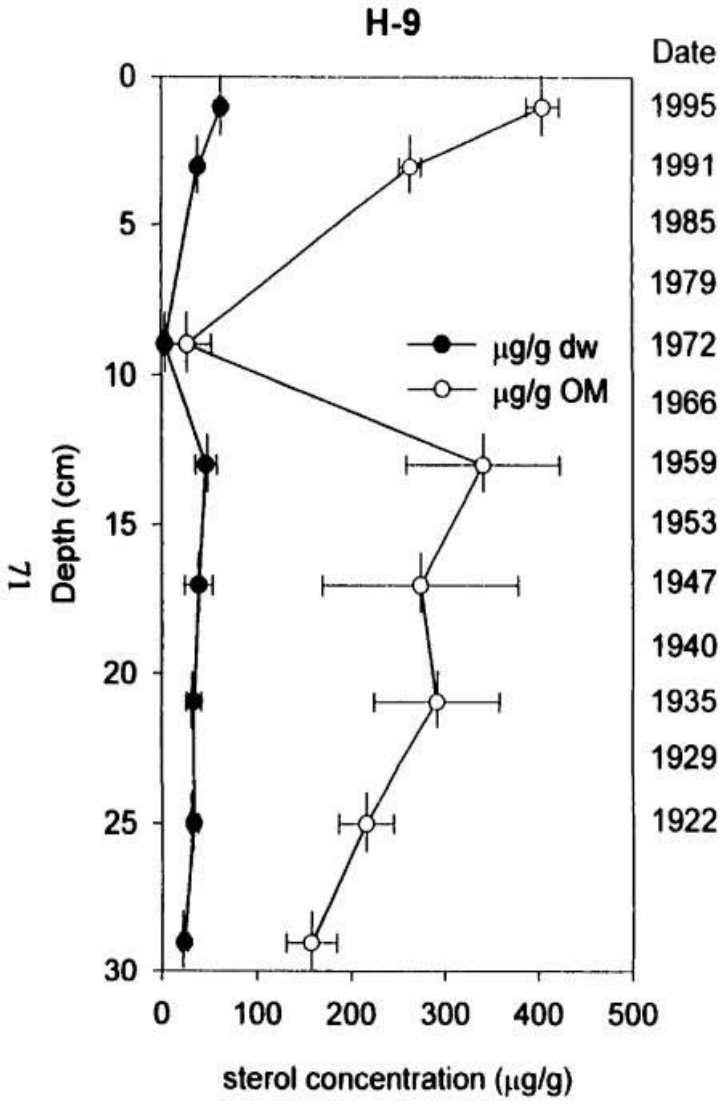
and 0.70 at H-9) than at St-7, indicating better preservation and suggesting preferential preservation of terrestrial sterols in marine sediments (as shown by Jasper and Gagosian, 1993).

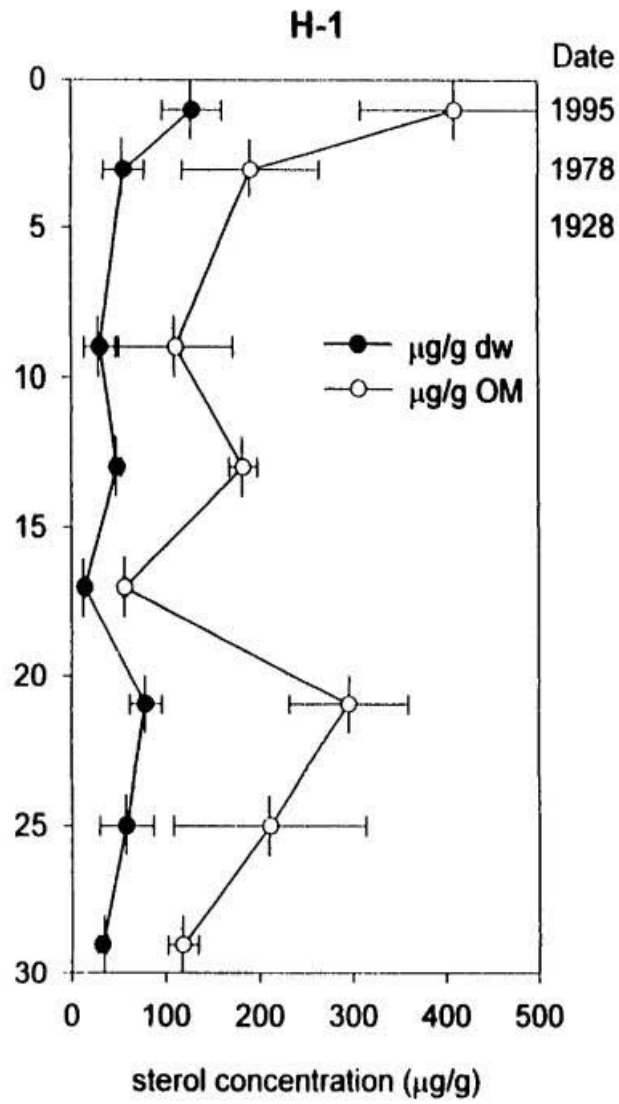
Certain sterols were not detected at all sites. Fucosterol is absent from H-1 sediments, and may reflect that marine algae are a relatively unimportant contributor to sedimentary sterols at this site. Ethylcoprostanol was detected only at H-1, possibly due to livestock manure input (Nichols *et al.*, 1996) from farming on Random Island. A sterol tentatively identified as 4,24-dimethyl-5α-cholest-22*E*-enol is likely of algal origin. 4-Methylcholestanol (likely of dinoflagellate origin, given the 4-methyl substituent) occurred only at H-9, and only in 3 horizons. Percentages of brassicasterol and stigmasterol were constant across all sites.

3.3.4.2 Historical (downcore) variation

At all core locations, surface sediments (0-2 cm) contained significantly more total free sterols (μg/g dw) (Table 3.3) than subsurface sediments, although the surface-subsurface difference at the off-shore site (St-7) disappears when these concentrations are normalized to the organic matter content. At no site did the percentage contribution of sterols to total lipids differ significantly from surface to subsurface sediments, suggesting that sterols are well preserved during burial, in contrast to the 66% loss in the top 3-5 cm of sediments noted in the Laurentian Trough (Colombo *et al.*, 1997). This may be due to the sediments being anoxic, as evidenced by their high elemental sulfur content (Favaro, 1998). Colombo *et al.* (1997) noted that the surface sediments of the cores they studied were bioperturbed (mixed

Figure 3.10. Total free sterol contents of sediments in individual depth horizons at in-shore stations. Mean ± SD, n = 3 analyses per depth. Vertical error bars indicate the 2-cm depth increments into which the core was sectioned for analysis.


H-9
Date
1995
1991
1985
1979
1972
1966
1959
1953
1947
1940
1935
1929
1922
μg/g dw
μg/g OM
Depth (cm)
0
5
10
15
20
25
30
0
100
200
300
400
500
sterol concentration (μg/g)


H-1
Date
1995
1978
1928
μg/g dw
μg/g OM
0
5
10
15
20
25
30
0
100
200
300
400
500
sterol concentration (μg/g)

by the action of benthic organisms).

At H-9, the bottom of the core (24-26 cm deep) dates from approximately 1922. ^{210}Pb dates suggested a far slower sedimentation rate at H-1, with material from 1928 being encountered at just 4-6 cm depth. It is not known whether this rate of deposition also holds for the material lower in the core. The St-7 core showed evidence of extensive bioturbation and ^{210}Pb dating was unable to establish a clear age sequence with depth. (St-7 sediments still had a high elemental sulphur content, suggesting that they are nonetheless an anoxic environment.) Depth profiling therefore focused on the in-shore sites H-9 and H-1. Downcore total sterol profiles had unusual minima at 16-18 cm deep (H-1) and 8-10 cm deep (H-9), regardless of how the sterol content is expressed (Fig. 3.10), suggesting declines in productivity or increased fluxes of inorganic material at these points.

Few downcore trends in sterol composition could be discerned, suggesting good preservation of the material during burial and no major changes in sources of organic matter in the recent past. At H-9, the combined contribution of 24-ethylcholesterol, 24-ethylcholestanol, ethylcholesta-5,22-dienol, 24-methylcholesterol and 24-methylcholestanol, has a pronounced minimum at 10-12 cm depth (Fig. 3.11a), suggesting a relative decrease in terrestrial input at that time, and a maximum at the bottom of the core (pre-1920's). Downcore alkane distributions at H-9 (Favaro, 1998) reflect the early twentieth-century sawmill boom in the area and a re-intensification of woodcutting in the 1950's. The observed terrestrial sterol minimum may reflect the period between these two phenomena.

Similarly, at H-1, the small maxima in the percentages of 24-ethylcholesterol and 24-

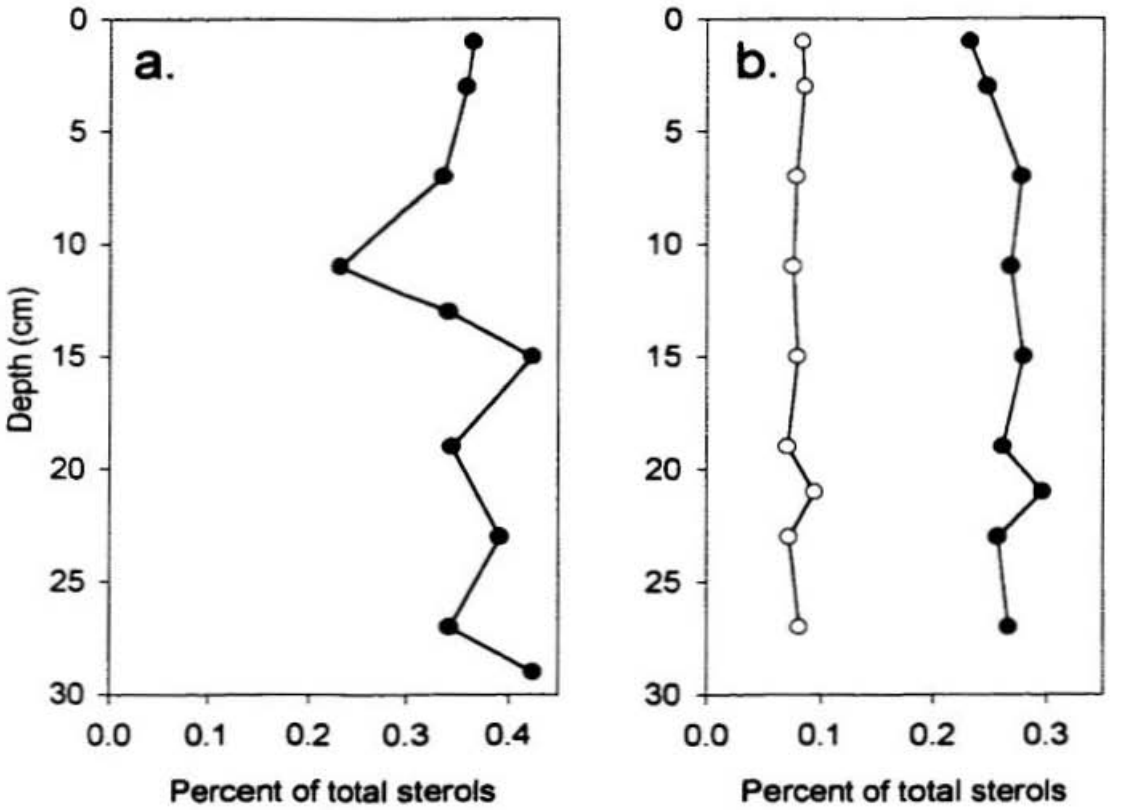


Figure 3.11. a. Downcore combined percentages of 24Et-29Δ^5, 24Et-29Δ^0, 24Et-29$\Delta^{5,22E}$, 24Me-29Δ^5 and 24Me-29Δ^0 at H-9. b. Downcore percentages of 24Et-29Δ^5 (●) and 24Et-29Δ^0 (○) at H-1.

ethylcholestanol at 20-22 cm depth suggest an increase in terrestrial input, which is corroborated by the abundance of carbohydrates in this horizon (Pulchan *et al.*, manuscript in preparation) (Fig. 3.11b). The stanol/stenol ratios for (*epi*)brassicastanol, cholestanol and 24-methylcholestanol all co-vary below 4-6 cm depth, suggesting that terrestrial sterols may not in fact be preferentially preserved in these sediments, although the 24-ethylcholestanol/24-ethylcholesterol ratio did not co-vary with these ratios.

3.3.5 Sterol-based source attribution of organic matter in Trinity Bay

Based on the preceding discussions of sterols in the plankton, settling particles and sediments, sources of these sterols in each sample type are assigned as noted in Table 3.5, which allows organic matter in these materials to be apportioned according to its origin (Fig 3.12). Cholesterol (and cholestanol) presented some problems in source assignment, being a major sterol in several of the diatom species detected, in dinoflagellates (Patterson, 1991), and in zooplankton. Furthermore, two of the common macroalgae of the area, *Ptiloda serrata* and *Rhodomela confervoides* (Pulchan *et al.*, manuscript in preparation) are Rhodophyceae and have cholesterol as their major sterol (Patterson, 1991). Thus, in sediments, a quarter of the cholesterol and cholestanol contributions were assigned to each of diatoms, dinoflagellates, zooplankton and macroalgae. In settling particles and plankton, in which no typical dinoflagellate sterols were detected, cholesterol and cholestanol were divided among the other three sources. Note that all these contributors are marine. 5β-stanols are grouped separatedly, since even naturally occurring ones are formed by bacterial

Table 3.5. Attribution of sterols in Trinity Bay samples to various sources of organic matter

Source	*Sterols attributed to source*
Higher plants	24-ethylcholesterol, 24-ethylcholestanol, 24-methylcholesterol, 24-methylcholestanol, ethylcholesta-5,22-dienol, ethylcholest-22-enol, 24-ethylcholestanol, C_{30} steratrienol
Phytoplankton (diatoms)	cholesterol, cholestanol, desmosterol, (*epi*)brassicasterol, (*epi*)brassicasterol, 24-methylenecholesterol, 24-methylenecholestanol, dimethyldehydrocholestanol
Dinoflagellates	dinosterol, dehydrodinosterol, 4-methylcholesterol, dimethyldehydrocholesterol, cholesterol, cholestanol
Zooplankton	*trans*-22-dehydrocholesterol, *trans*-22-dehydrocholestanol, 24-nordehydrocholesterol, 24-nordehydrocholestanol, cholesterol, choiestanol, occelasterol
Macroalgae	fucosterol, fucostanol, isofucosterol, isofucostanol, cholesterol, cholestanol
5β-stanols	Coprostanol, *epi*coprostanol, ethylcoprostanol

biohydrogenation (see Section 3.3.6).

The higher plant contribution to the sedimentary material contrasts clearly with the net-tow and settling particle samples, to which diatoms and zooplankton are the major contributors (Fig. 3.12). Higher plant material makes the greatest contribution at H-1 (61% of sedimentary sterols, but even at off-shore St-7, at 363-m water column depth, higher plant material is still the greatest contribution (31%). Venkantesan *et al.* (1987) observed typical phytosterols in the North Atlantic off the New England coast in sediments from depths greater than 1000 m. Thus, anthropogenic effects on the amount of terrestrial input may be reflected in sediments far into the oceans.

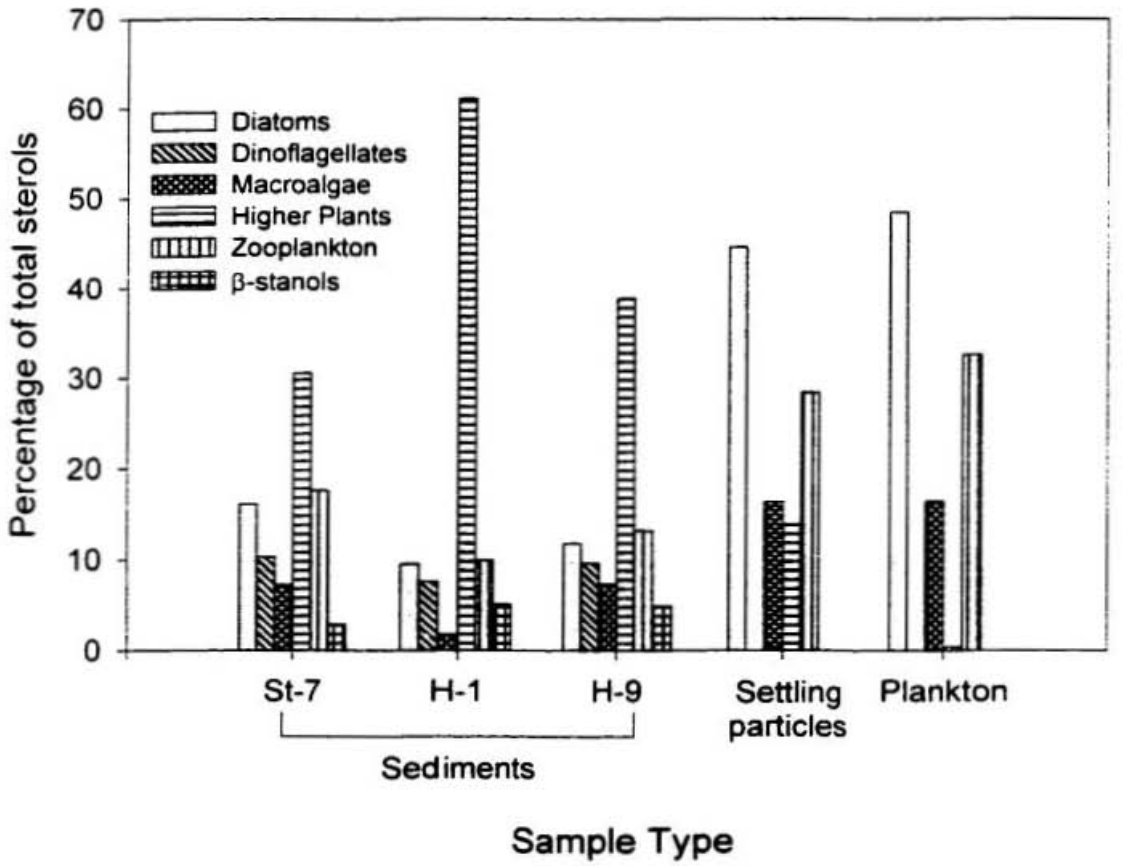


Figure 3.12. Sources of organic matter in Trinity Bay plankton, settling particles and sediments according to sterol biomarker composition. Sediments, n=8-10; settling particles, n=9; plankton, n=10.

3.3.6 Fecal biomarkers

No 5β-stanols or stanones were detected in sediment trap and net tow samples. Coprostanol and *epi*coprostanol elute close together and have identical mass spectra. Selected St-7 sediment trap samples and core extracts were spiked with authentic standards of *epi*coprostanol, coprostanol or both and analyzed by GC-FID at three different carrier gas head pressures in order to influence elution time and distinguish these compounds from co-eluting substances. Small quantities of *epi*coprostanol are present in St-7 sediments; no coprostanol was detected. Certain core horizons from H-1 and H-9 contained coprostanol and/or *epi*coprostanol as minor components.

The data suggest that, despite the universal practice of discharging sewage into coastal waters from small communities in the Trinity Bay area, the quantities discharged are insignificant or dispersal and degradation of the material is efficient. Alternatively, a fecal sterol signature in sediments may be highly localized near sewage out-falls, since coprostanol is strongly associated with particulate matter (Pierce and Brown, 1984), and its concentration may therefore drop sharply away from a discharge point. Since the original coring sites were not chosen based on distance to sewage out-falls, it is possible that a sedimentary sewage signature is present but restricted to the immediate vicinity of discharge sites.

3.3.6.1 St. John's Harbour sediment

To provide a comparison with an environment known to be severely polluted, as well as to confirm that the method used was a reliable one for detecting coprostanol, sediment

from St. John's harbour was analyzed. The harbour is a largely enclosed basin (maximum depth 32 m, with a 12-m deep entrance sill: Reid, 1998) which receives untreated sewage waste from an urban population of 130,000 (St. John's Harbour ACAP, 1996). A 29.92 g (dw) sample of sediment (collected in the centre of the harbour (29 m depth) just inside the entrance straight (the Narrows) using a pre-cleaned Van Veen grab sampler: O'Malley, 1994) was extracted as described (Section 2.3). Of the extract, 10 μL was saponified, extracted and derivatized as above, made up to 100 μL with hexane containing 0.048 mg/mL cholestane, and analyzed by GC/MS.

Coprostanol was the major sterol in the sediment (semi-quantitative determination, approx. 20 μg coprostanol/g dw). This confirms substantial contamination: Writer *et al.* (1995) reported up to 7.5 μg/g in sediments from contaminated Mississippi locks, while Pierce and Brown (1984) measured 2.5 μg/g in a Sarasota, FL, polluted Bayou. While no spike was added to differentiate between coprostanol and *epi*coprostanol, it is assumed that marine mammals do not contribute appreciably to the sterols in St. John's Harbour sediments, nor is effluent discharged into the harbour treated with sludge digesters. No cholestanol was detected, and the ratio 5β / (5α + 5β) for C_{27} sterols is therefore 1. The co-elution of peaks due to m/z 316 and 231 (mass spectral fragments for coprostanone: Venkantesan and Santiago, 1989) in an extracted ion chromatogram suggest that coprostanone, which was not detected in any other samples, is also present, although wholly buried beneath the coprostanol peak.

In addition to coprostanol, cholesterol, 24-ethylcoprostanol and 24-ethylcholesterol

were the principal sterols in the St. John's Harbour sediment. The 24-ethylcholesterol is consistent with an enclosed harbour receiving substantial terrestrial run-off. 24-Ethylcoprostanol would originate either from livestock manure (Nichols and Espey, 1991; Bull *et al.*, 1998), in which its formation is analogous to the formation of coprostanol from cholesterol, or from sedimentary Δ^5-hydrogenation of the terrestrial 24-ethylcholesterol. If the latter were the case, some sedimentary coprostanol may also originate this way. However, the ethylcoprostanol/ethylcholesterol ratio (Quemeneur and Marty, 1992) is 0.74 (again assuming equal detector responses for all sterols), lower than the coprostanol/cholesterol ratio (2.0). Furthermore, livestock manure from farms along the Waterford River, the principal river draining into the harbour, may be a source of ethylcoprostanol. These two lines of evidence point to sewage as the principal source of coprostanol in the harbour sediments. The unidentified C_{30}, Δ^5 component prominent in sediments at H-9 was also detected, supporting its postulated terrestrial origin.

3.4 Sterols in Newfoundland marine samples: suggestions for future approaches

The data illustrate both the potential and some of the difficulties of using sterol biomarkers to identify sources and transformations of organic material. Several other approaches could be used to further elucidate these processes. Firstly, marine and terrestrial C-24 α and β sterol epimers could be differentiated. While C-24 stereochemical analysis on non-polar columns is prohibitively lengthy (Maxwell *et al.*, 1980), the use of a chiral cyclodextrin GC column, recently available in our laboratory, to analyze specific C-24

alkylsterols is an exciting possibility. Secondly, compound-specific carbon isotope analysis, which can distinguish terrestrial from marine material on the bases of characteristic $\delta^{13}C$ values, has been successfully linked with sterol molecular species analysis (Jasper and Gagosian, 1993), and could be conducted on these samples, although scrupulous care is required to avoid isotope fractionation artifacts during the many sample preparation steps. Finally, the classical saponification-derivatization method used does not differentiate free sterols from steryl esters, and does not detect sterols in polar conjugates. The biogeochemical information available by differentiating these species warrants development of new methods for their facile analysis, as described in Part II.

Part II: Development of Thin layer chromatography-Pyrolysis-Gas chromatography/Mass spectrometry

4. Experimental

4.1 Chemicals and glassware

Glassware was treated as described in Section 2.1. All solvents used for rinsing, dilution and development were trace analysis grade (Omnisolv, BDH or Optima, Fisher) except diethyl ether (ACS grade, delivered in glass, BDH) and acetone (Spectrograde, Caledon). Nanopure water, triple-extracted with Omnisolv chloroform, was used in developments. Lipid standards were purchased as individual pure compounds from Matreya (Pleasant Gap, PA) (steryl glucoside), Steraloids (Wilton, NH) (certain sterols: Section 2.1) and Sigma (St. Louis, MO) (all other lipids).

4.2 Marine samples

As part of a study of marine lipids in Newfoundland coastal waters, settling particles were sampled using sediment traps moored for 28 days at 75 and 100 m depths at St-7 (Fig. 1.6) in Trinity Bay during April 1995. The material was extracted as previously described (Section 2.2) and the TLE stored under N_2 at -20°C until analysis.

4.3 Hydrogenation of total lipid extracts

Aliquots (1.5 mL) of the TLEs of the sediment trap samples were hydrogenated with ~5 mg of platinum (IV) oxide catalyst (Sigma, St. Louis, MO) in 4-mL vials. The method used has been shown to be equivalent to hydrogenation on commercial atmospheric pressure apparatus (Yang *et al.*, 1996). A gentle stream of hydrogen was bubbled through the extracts *via* stainless steel 18-gauge needles piercing loosely-fitted, Teflon-faced septa for 20 minutes. The needles were withdrawn and the extracts stirred vigorously for 6 hours. After catalyst removal (pre-rinsed Whatman #2 filter paper) and washing and extract evaporation under HP^+-grade N_2, the samples were re-diluted to 0.3 mL in chloroform.

4.4 Chromarod development and scanning

Solutions were spotted onto the Chromarods-SIII below the origin using a 25-μL blunt-tipped #705 syringe with a repeating dispenser (Hamilton Co., Reno, NV) (for all samples and standards to be developed) or a 10-μL #701 syringe (for most standards desorbed without development). Prior to development, racks of Chromarods were dipped in acetone until the solvent front reached the origin, a process repeated twice to focus spotted material. The rods were treated with a three-development, three-scan procedure (Fig. 4.1) reported by Parrish (1987). The first developments in hexane: diethyl ether: formic acid (99:1:0.05) separated hydrocarbons, wax and steryl esters, fatty acid methyl esters (FAMEs)

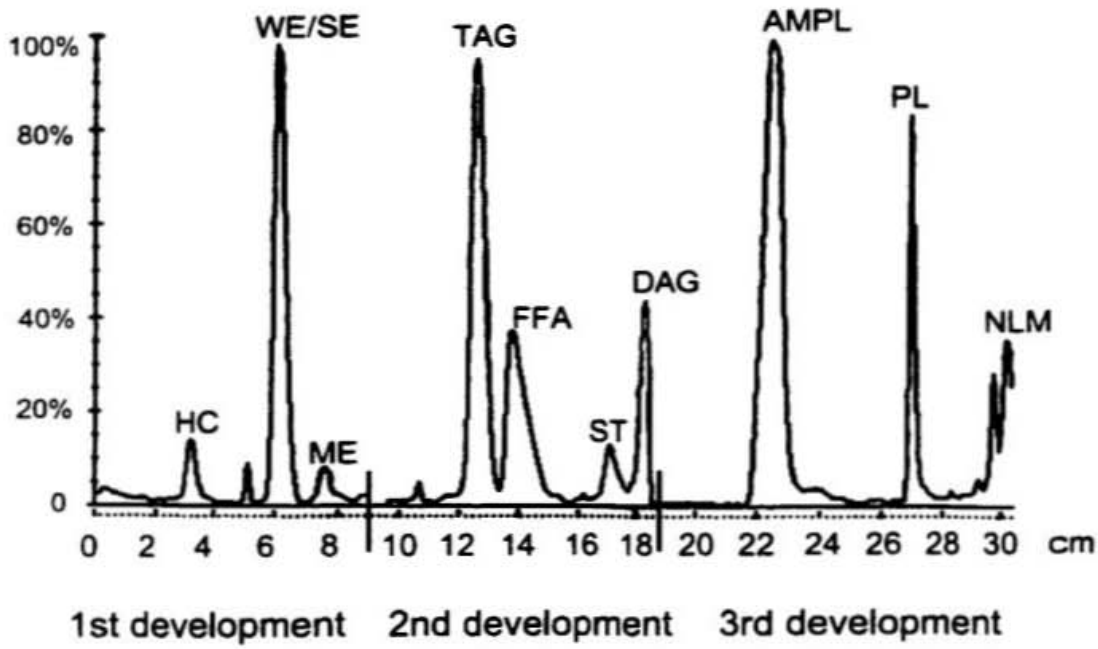


Figure 4.1. Typical Iatroscan TLC-FID chromatogram: hydrogenated TLE of settling particles (St-7, 75 m depth), showing three developments and scans. Development is from right to left. HC- Hydrocarbons; WE/SE- Wax & steryl esters; ME- (Fatty acid) methyl esters; TAG- triacylglycerols; FFA- Free fatty acids; FS- Free sterols; DAG- diacylglycerols; AMPL- Acetone-mobile polar lipids; PL- phospholipids; NLM- Non-lipid material. No aliphatic ketones (KET) or alcohols (ALC) were detected.

and ketones. This was followed by a partial scan to 23 mm above the origin. The second development, in hexane: diethyl ether: formic acid (80:20:0.1), separated triacylglycerols, free fatty acids, aliphatic alcohols, free sterols and diacylglycerols. This was followed by a partial scan to 11 mm above the origin. The final developments in 100% acetone and chloroform: methanol: water (5:4:1), eluted the acetone-mobile polar lipids (AMPL) and phospholipids and was followed by a full scan to below the origin.

4.5 Desorption-gas chromatography-mass spectrometry

A Pyroprobe 120 platinum coil pyrolyzer and GC interface (Chemical Data Systems, Oxford, PA) was used to desorb lipids from the Chromarod coating and introduce them onto a GC column. The interface was mounted on a HP 5890 II Gas Chromatograph /5971A Mass Selective Detector (Hewlett-Packard, Palo Alto, CA), fitted with a 25 m x 0.25 mm I.D. 100% polydimethylsiloxane column (as per Section 2.4). The mass spectrometer was operated in the E. I. mode (70 eV). Sections of Chromarod (1-2 cm) were placed in a quartz tube in the platinum wire coil of the pyrolysis probe (Fig. 4.2). After introduction of the probe into the heated interface, re-sealing of the system, and the restoration of carrier gas pressure the probe was flash heated to its set temperature (Table 4.1). An initial flow of 5 mL min^{-1} (1 min) was used to sweep desorbed material rapidly onto the column, after which the flow was dropped to 1 mL min^{-1}. The carrier flow was in split mode (approximate split ratio 20:1). Two temperature programs were generally used: Program A (50°C for 2 min,

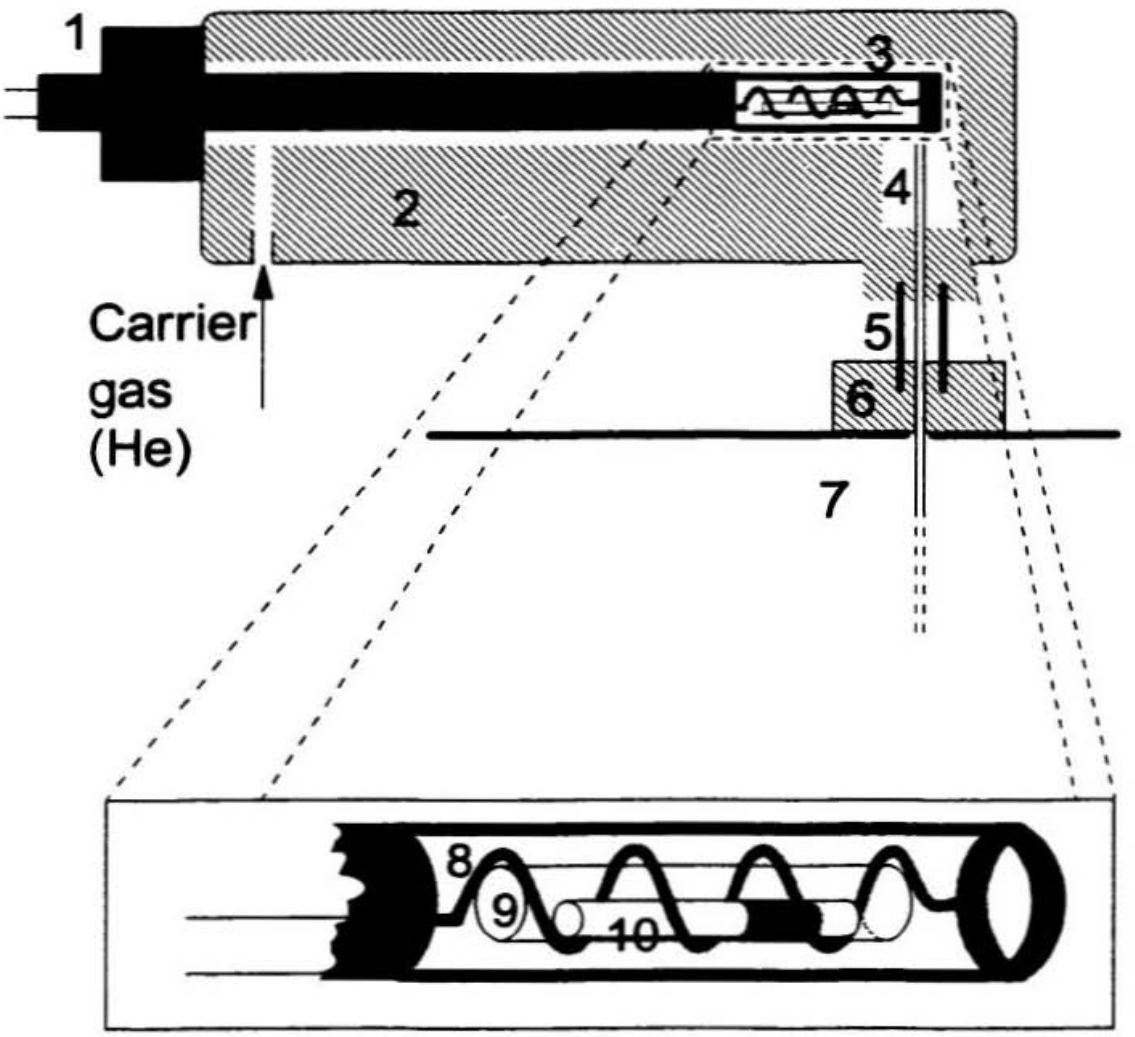


Figure 4.2. Pyroprobe and interface used to desorb/pyrolyze lipids off silica Chromarod coating. 1. Pyroprobe with Pt coil; 2. Heated interface; 3. Probe tip (see detail); 4. Capillary GC column threaded into interface; 5. Steel transfer tube; 6. Modified injector port nut; 7. GC oven; 8. Pt heating coil; 9. Quartz tube; 10. Chromarod section with lipid band.

Table 4.1. Conditions used to transfer lipids from Chromarod coating to GC column by thermal desorption/pyrolysis.

Lipid classes desorbed without further treatment		
Lipid class	Interface Temperature* (°C)	Pyroprobe (coil) Temperature Setting (°C)
Hydrocarbons	Low	300
Wax esters & Steryl esters	High	450
Fatty acid methyl esters	Low	400
Aliphatic ketones	Low	400

Lipid classes desorbed after trimethylsilylation on the Chromarod coating		
Lipid class	Interface Temperature* (°C)	Pyroprobe (coil) Temperature Setting (°C)
Free fatty acids	Low	300
Aliphatic alcohols	Low	300
Free sterols	Low	300/400
1-Monoacylglycerols	High	500
Steryl glucosides	High	500

Lipid classes desorbed by thermochemolysis with TMAH		
Lipid class	Interface Temperature* (°C)	Pyroprobe (coil) Temperature Setting (°C)
Triacylglycerols	Low	450
Phospholipids (PC & PE)	Low	250
Steryl glucosides	High	600

* Measured temperatures in the Pyroprobe interface were Low- 250 °C, High- 300 °C. Reproducible to within ±15°C

30°C/min to 200°C, 5°C/min to 300°C, 5 min hold) for steryl esters, wax esters and free sterols (both underivatized and as TMS ethers), and Program B (50°C for 2 min, 10°C/min to 300°C, 5 min hold) for MAGs, aliphatic alcohols, hydrocarbons, ketones, fatty acid TMS esters, and FAMEs, as well as for any bands from marine samples where compounds with a large range of molecular weights were anticipated.

Initial experiments involved desorption of standards (1-10 μL) spotted onto 1-2 cm sections of Chromarods and focused into a narrow band near one end of the section by dipping an end into acetone or chloroform after spotting. The rod fragment was placed in the coil such that band of material was at the end closest to the column head (Fig. 4.2).

To isolate individual lipid bands for desorption, the Chromarods were etched with a knife and broken by holding tightly with forceps. The bands were located based on FID scans of adjacent rods spotted with the same sample. Where the band required for desorption eluted in the second or third development, material from prior development was removed from the rod by combustion in the Iatroscan Mk V.

4.6 Trimethylsilylation

For certain lipid classes containing free hydroxyl or carboxyl groups, trimethylsilyl ethers or esters were formed by applying 2-3 μL BSTFA + 1% TMCS to the material on the Chromarod surface. The rod fragment was then heated in a foil-covered 2-mL vial (60 °C, 10-15 min) before being placed in the Pyroprobe coil for desorption/pyrolysis.

4.7 Thermochemolysis

To convert involatile acyl lipids directly to the corresponding FAMEs by thermochemolysis, 0.24 % (w/v) methanolic tetramethylammonium hydroxide (TMAH, pentahydrate, Sigma) was applied to the bands on Chromarod sections before pyrolysis in the pyroprobe set at 500 or 600 °C (Table 2.1). While this represented a large TMAH excess, much of it may be removed from the rod by thermal decomposition in the heated interface while the carrier gas pressure is being restored. In later experiments, 5 % methanolic TMAH was therefore used. This was the highest concentration used because prolonged use of TMAH leads to gradual corrosion of Vespel parts of the GC's pressure regulation apparatus downstream of the injector port.

5. Results and discussion

5.1 Hydrogenation

Hydrogenation of TLE's is valuable in TLC-FID lipid analysis, giving improved peak shapes, increasing response, and decreasing peak splitting which may arise due to different degrees of unsaturation within a lipid class (Shantha and Ackman, 1990). Furthermore, the stability of lipid extracts to oxidation is greatly improved by saturation of the double bonds (Shantha and Ackman ,1990; Shantha, 1992). Here, hydrogenation gives a clearer picture of the acyl carbon number distribution of settling particulate lipids after TMAH thermochemolysis (Fig 5.1). Furthermore, tailing of the wax ester peaks is decreased, presumably due to the existence of fewer molecular species within each peak. This approach is particularly useful where many species are expected in a certain lipid class band, with each individually being below the detection limit. Hydrogenation effectively combines these species into fewer peaks, while still allowing useful carbon number information to be obtained. Yang *et al.* (1996) also used this approach to improve resolution in the direct GC analysis of marine neutral lipids. The persistence of some wax esters in these chromatograms also illustrates that low concentrations (0.24% w/v) of TMAH (Fig. 5.1a) are not sufficient for complete thermochemolysis of acyl lipids and that even at higher concentrations (5% w/v) (Fig. 5.1b) small amounts of wax ester remain unreacted.

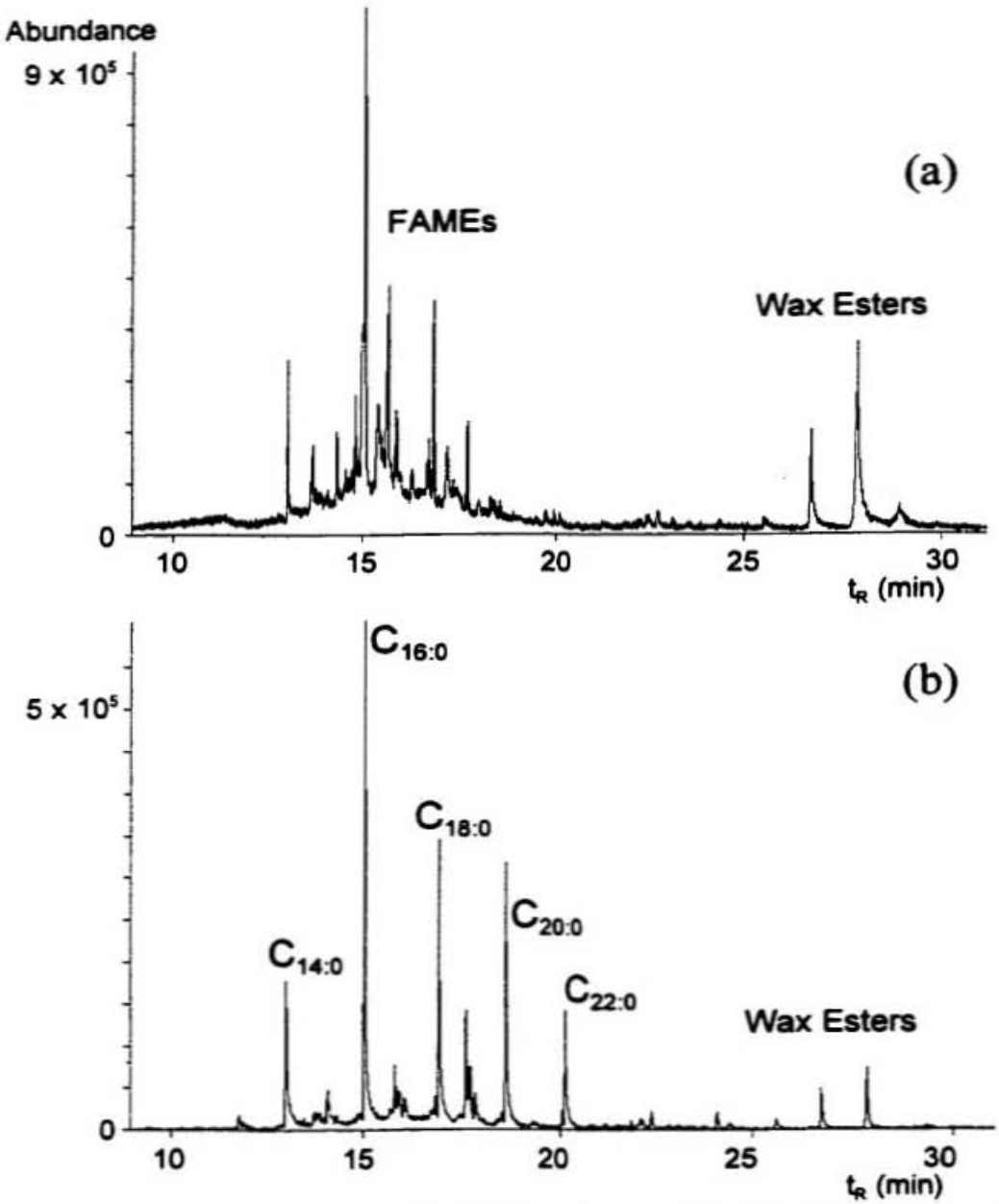


Figure 5.1. Effect of hydrogenation of the TLE (settling particles, 75 m): thermochemolysis with methanolic TMAH of: (a) Original TLE (42 μg total lipid spotted on rod); (b) Hydrogenated TLE (14 μg total lipid).

5.2 Neutral lipid classes

5.2.1 Steryl and wax esters

Two cholesteryl esters (cholesteryl palmitate, CP, and cholesteryl arachidate, CA) both yielded a pyrolysis product (cholesta-3,5-diene), presumably due to deacylation (Fig. 5.2). The intact steryl esters were not observed, nor were any clearly recognizable fragments attributable to the acyl moieties. Intact steryl esters can be determined at high temperatures ($\leq$ 370°C) on long columns (Wakeham and Frew, 1982) or on short (5 m) colums (Yang *et al*, 1996). However, due to their special column requirements, neither of these approaches would be of general utility for the range of lipid classes attempted in this work. Since our interest was mainly in the sterol moiety of steryl esters, the present approach was adopted.

Wax esters were desorbed from the Chromarod surface intact, although roughly equal masses of the 16:0/16:0 (0.5 μg), 18:0/16:0 (0.5 μg) and 18:0/18:0 (1 μg) esters showed that GC-MS peak area decreased with increasing mass (Fig. 5.2). It is not known whether this is due to greater thermal degradation of larger esters, or less efficient desorption or transfer out of the pyroprobe interface.

The wax ester/steryl ester band of hydrogenated particulate lipids (75 m) revealed that the contribution from steryl esters is small (~8% of the total band), and due only to C_{27} sterols (cholestadiene peak) (Fig. 5.3). The wax ester carbon number distribution increases from C_{30} to C_{36}, above which no other wax esters are observed. This distribution differs not only from the more normal one reported by Wakeham and Frew (1982), but also from that

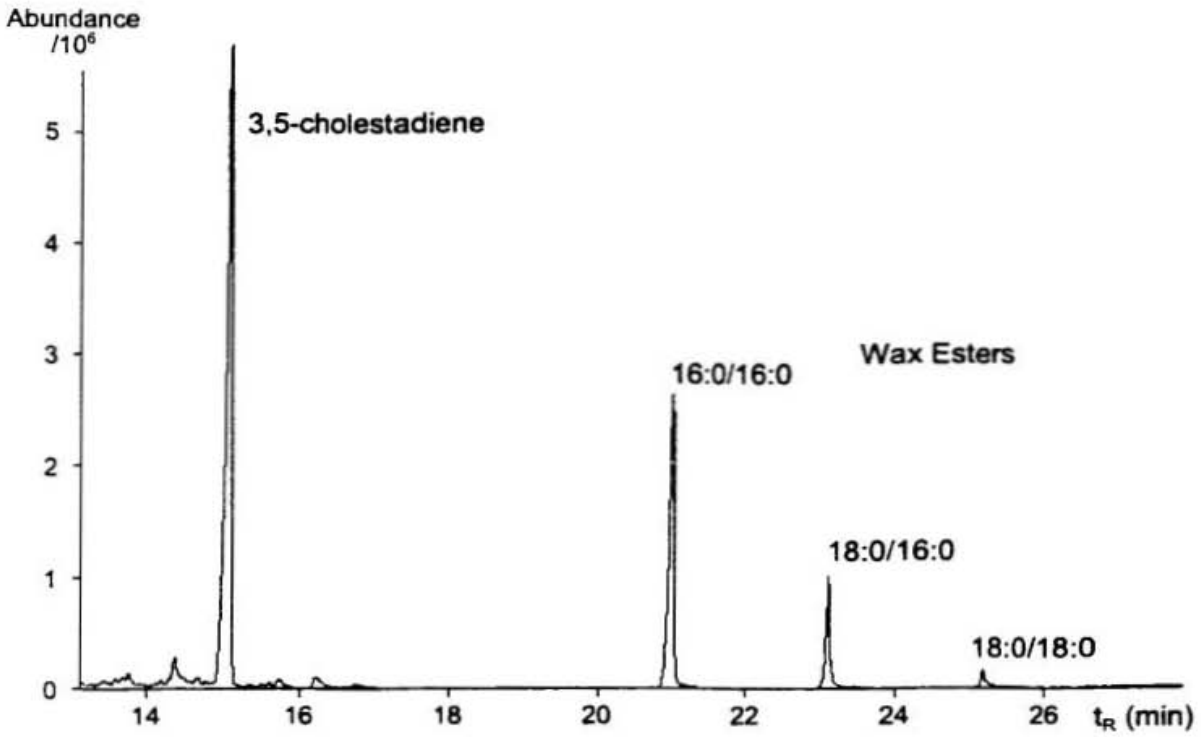


Figure 5.2 Three wax esters (16:0/16:0, 18:0/16:0, and 18:0/18:0) and two cholesteryl esters (cholesteryl palmitate and arachidate) desorbed off a Chromarod section by a Pyroprobe set to 450 °C. 3.1 μg total lipid.

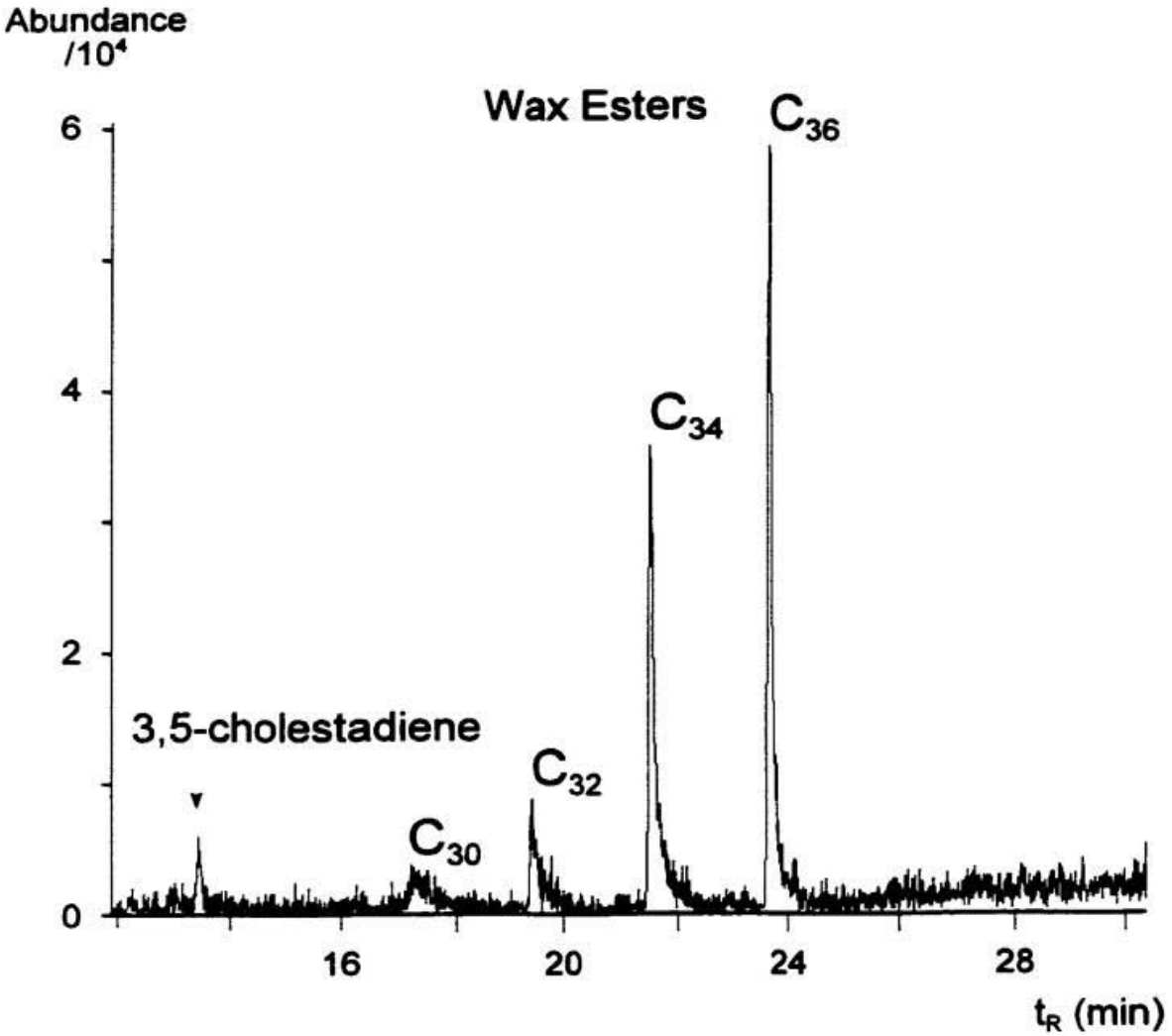


Figure 5.3 Wax ester/steryl ester band from hydrogenated TLE of settling particles (St-7, 75 m depth) desorbed from Chromarod.

of wax esters detected by desorption of spotted, unseparated material (14 μg TLE), in which wax esters with molecular masses up to 564 amu (C_{38}) were detected. At a higher sample load (42 μg TLE) on an undeveloped rod section, traces of the wax esters up to 620 amu (C_{42}) were also detected, although spotting this much material reduces TLC resolution. When the Chromarods are developed, each individual wax ester may not be uniformly distributed throughout the band, and if the full band is not included in the fragment cut from the rod, this may bias the distribution of wax esters seen by desorption. The application of the method to specific regions of an intact rod would be a major step towards alleviating this problem.

The mass spectra corresponding to each GC peak contained prominent $[RCO_2H_2]^+$ ions of several chain lengths (where R is derived from the wax ester acyl moiety), demonstrating that each carbon number (Fig. 5.3) contains several alkyl/acyl combinations (Wakeham & Frew, 1982). Assuming equal ionization efficiencies for all of these, the C_{36} wax ester is mainly C_{22}/C_{14} (80%), with some contribution from C_{20}/C_{16} (17%). The C_{34} peak contained, in decreasing order of abundance, C_{20}/C_{14} (75%), C_{16}/C_{18} (16%), and C_{18}/C_{16} (6%), while C_{16}/C_{16} (57%), and C_{14}/C_{18} (37%) predominated among the C_{32} esters. These are in good agreement with the ratios determined for each peak in the undeveloped extract. The C_{22}/C_{16} ester was the most common C_{38} species. In settling particles from the highly productive Peru upwelling, C_{16} and C_{18} acids (31 % and 46 % of total, respectively) and C_{16} alcohols (64 % of total) were the most common of the wax ester constituents (Wakeham and Frew, 1982). The wax esters of calanoid copepods contain mainly C_{16}, C_{20} and C_{22} saturated and monounsaturated fatty alcohols (Sargent *et al.*, 1981)- *Calanus finmarchicus*, an

important copepod in Newfoundland waters (B. deYoung, *pers. comm.*), contains C_{14}-C_{22} fatty acids and predominantly (90% of total) 16:0, 20:1 and 22:1 alcohols (Kattner, 1989). Thus, the alkyl/acyl combinations observed suggest a zooplankton source of wax esters in these settling particles. Wax esters are the main storage lipids of many cold water zooplankton, and few marine algae produce significant amounts of wax esters.

The detection of only C_{27} sterols in the band is consistent with a marine source for steryl esters (Volkman, 1986), and indicates that the carbon number distribution of sterols in steryl esters mirrors that in free sterols in these particles (Section 3.3.2). While the minor (8 %) contribution of steryl esters to the steryl ester/wax ester band confirms that this band is a reliable indicator of zooplankton wax ester input, this 8 % is appreciable in comparison to the free sterols themselves. For the sample discussed in Fig. 5.3, steryl esters would be present at 20 % of the concentration of free sterols. For other samples containing more of the wax ester/steryl ester band, the contribution of steryl esters to total sterols may be greater still. Steryl esters comprised <10 % of total sterols in settling particles in a shallow marine inlet (Pocklington *et al.*, 1987) and <5% of total sterols in particulate matter in the open tropical Atlantic (Wakeham *et al.*, 1980).

5.2.2 Free sterols

Desorption of 3.6 μg of a single sterol, cholestanol, at 300 °C produced the dehydrated sterol (2- or 4-cholestene) as the largest peak (Fig. 5.4a, A). This suggests that the sterenes could be used to identify the original sterol present, much as was done for steryl esters. However, appreciable amounts of the other sterene (Fig. 5.4a, B) and an unidentified C_{12} hydrocarbon (Fig. 5.4a, C), likely due to thermal decomposition of the sterol, were also consistently present. Moreover, as the amount of sterol applied to the rod section was decreased to 0.36 μg, the hydrocarbon fragment became the predominant peak. Desorption of a ten-sterol standard gave very low yields of any diagnostically useful sterenes, with various dehydration products instead forming a cluster of peaks (Fig. 5.4b, D) and with a high abundance of the unidentified hydrocarbon (Fig. 5.4b, E). Clearly, this approach would be untenable for the analysis of marine material, where 20 or more individual sterols may be present (Section 3.3). Treatment of the same standard with BSTFA after spotting gave clear peaks for sterol TMS ethers (Fig. 5.4c, F) and decreased the sterene contribution, although some individual sterols were not detected. Setting the pyroprobe to 400 °C desorbed the sterols entirely as their TMS ethers, with no sterenes detectable (Fig. 5.5).

The trimethylsilylated sterol band from a hydrogenated TLE of settling marine particles (100 m) showed a clear carbon number distribution, with C_{27} sterols most abundant, and smaller amounts of C_{28}, C_{29} and C_{26} sterols (Fig. 5.5). The TMS ether mass spectra revealed that hydrogenation had not reduced the Δ^5 double bond in the steroid nucleus, as

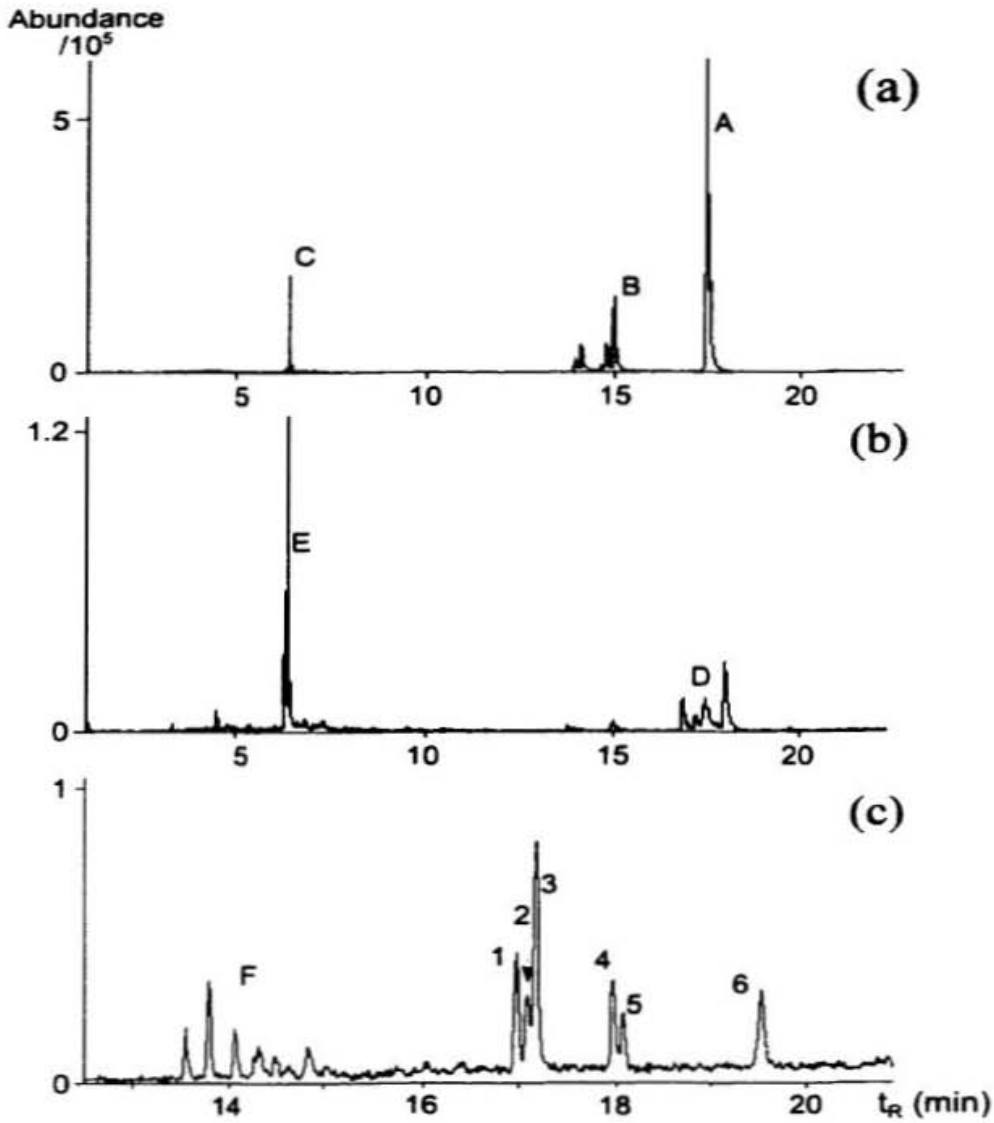


Figure 5.4. Desorption of free sterols; Pyroprobe set to 300 °C. (a) 3.6 μg 5α-cholestanol desorbed without further treatment. (b) Mixed sterol standard (9 sterols plus coprostanone, 1.3 μg total) desorbed without further treatment. (c) Mixed sterol standard (0.78 μg total), desorbed after addition of 10 μL BSTFA. TMS ethers: 1, coprostanol; 3, epicoprostanol; 4, cholesterol; 5, cholestanol; 6, lanosterol/fucosterol. 2: coprostanone. Sitosterol, campesterol and stigmasterol, while present in the standard, were not detected.

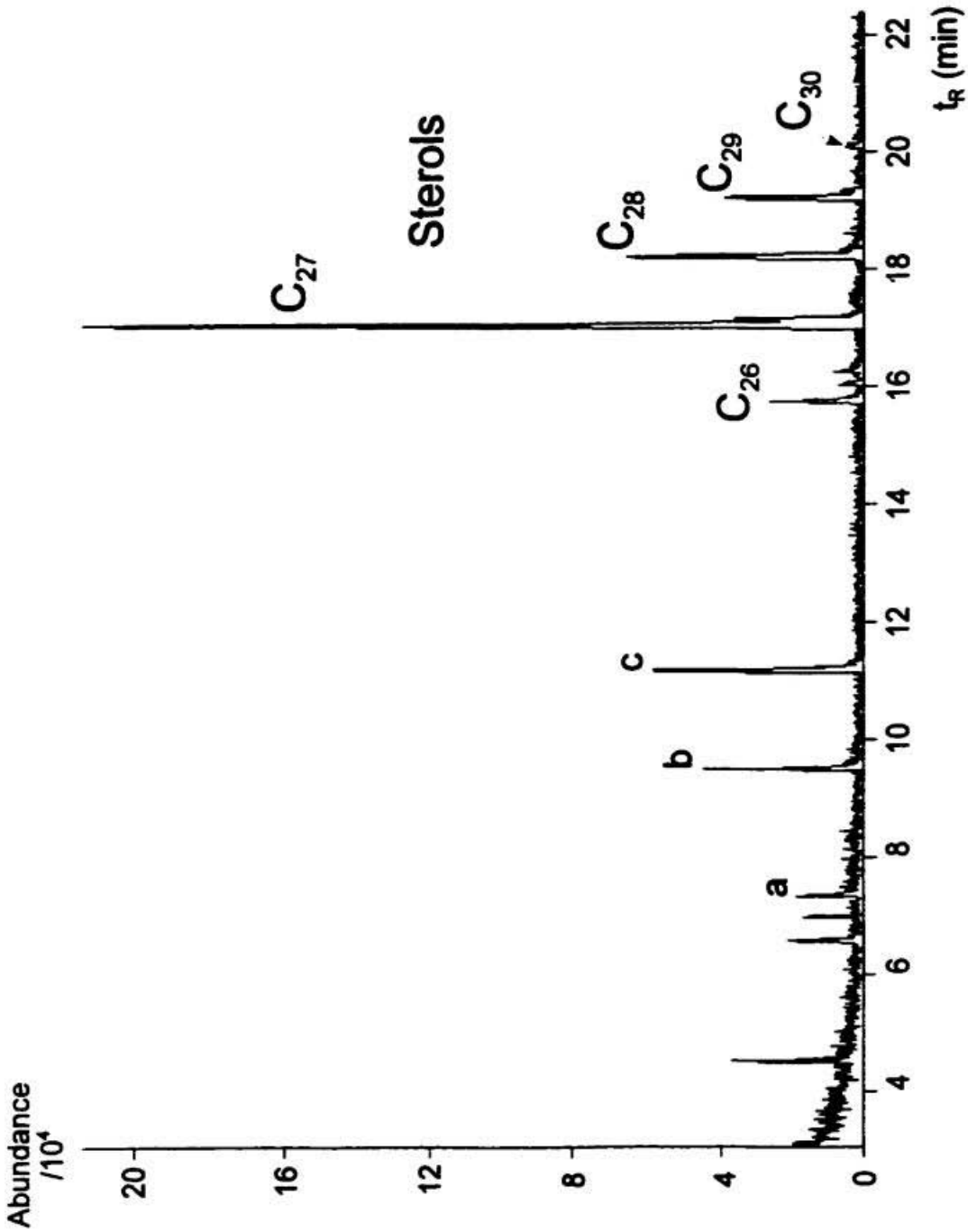


Figure 5.5. Free sterol band from hydrogenated TLE of settling particles (St-7, 75 m) desorbed from a Chromarod as TMS ethers. Pyroprobe set at 400 °C. Lettered peaks are aliphatic alcohol TMS ethers: a, C_{16}; b, C_{20}; c, C_{22}.

also found by Yang *et al.* (1996) using this procedure. Therefore the small amounts of cholestanol, 24-methylcholestanol and 24-ethylcholestanol in the chromatogram were present in the original unhydrogenated sample, an observation consistent with the saponification/ derivatization/GC results (Section 3.3). The prominence of C_{27} sterols in the sample also matches the prominence of C_{27} sterols (desmosterol and cholesterol) in settling particles and plankton. A very small peak corresponding to C_{30} sterols is also apparent, likely due to a dinoflagellate contribution. No C_{30} sterols were detected in settling particles or plankton analyzed by saponification/derivatization/GC, suggesting that dinoflagellates were more important contributors to the April 1995 sediment trap samples than to those from the periods discussed in Section 3.3. Alternatively, the C_{30} peak observed here may only have been detected because a single peak represents the entire C_{30} contribution. In contrast, the unhydrogenated sample may contain several C_{30} species, with each being below the detection limit.

5.2.3 Triacylglycerols

Desorption of triacylglycerol (TAG) standards illustrates the value of thermochemolysis with TMAH. Desorption of untreated TAGs was unsuccessful at Pyroprobe settings up to 600°C. Moreover, when tristearin (trioctadecanoylglycerol) was injected, it did not elute from the GC column used at a temperature compatible with the stationary phase (325°C) . De Luca *et al.* (1992) analyzed TAGs by Curie-point pyrolysis-GC-MS, reporting that they were desorbed largely intact at 610°C. However, a metal Curie-

point wire is not as porous or polar a surface as the silica coating of a Chromarod. Furthermore, these authors used very high temperatures ($\leq$ 390°C) in the GC separation, an approach incompatible with our aim to develop a technique applicable to many lipid classes and employing common, general-use columns.

Application of 10 μL of 0.24% methanolic TMAH to the triacylglycerols tricaprin (tridecanoylglycerol), tristearin and triarachidin (trieicosanoylglycerol) on a Chromarod section yielded capric (decanoic), stearic (octadecanoic) and arachidic (eicosanoic) acid methyl esters. Sensitivity differed with carbon number, with a pronounced decrease from the C_{18} FAME to the C_{20} FAME. This led to the subsequent use of ten-fold greater quantities of TMAH to ensure full thermochemolysis of the larger TAGs.

The C_{16} FAME was the most common in the TAG band from 75 m settling particles thermochemolysed with 5 μL of 5% methanolic TMAH, with C_{18} and C_{20} FAMEs nearly as abundant and smaller amounts of C_{14} and C_{22} FAMEs present.

5.2.4 Hydrocarbons

While standard *n*-nonadecane was readily desorbed by the Pyroprobe coil set at 350 °C after the first development, very few hydrocarbons could be clearly detected and identified in the settling particulate lipids at 75 or 100 m depths. Phytane (2,6,10,14-tetramethylhexadecane), a degradation product of algal pigments, was present, along with an earlier hydrocarbon peak with m/z 268, possibly pristane (2,6,10,14-

tetramethylpentadecane). The latter is a further breakdown product of phytane. No unambiguously anthropogenic or petroleum hydrocarbons were detected. Favaro (1998) reported that no significant anthropogenic hydrocarbon contamination was present in the sediments in the study area.

5.2.5 Free fatty acids

Palmitic and stearic (C_{16} and C_{18}) free fatty acids (FFAs) could not be desorbed directly from Chromarod sections by temperatures up to 270 °C. This is likely due to strong absorption of the carboxyl groups onto the polar silica. Conversion to their trimethylsilyl esters using BSTFA allowed both acids to be desorbed in good yields. This also held for palmitic acid in a multi-component standard following two TLC developments. In the free fatty acid band from the TLE of settling particles at 75 m depth, C_{16} acids predominated (65% of total, assuming equal MS detection efficiencies for all acids), with smaller amounts of C_{18} (13.5%) and C_{20} (5.5%) acids and minor amounts of C_{14} (2.1%), C_{15} (2.5%) and C_{17} (4.2%) acids also present. The two peaks with mass spectra consistent with a C_{15} acid TMS ester may be due to *iso* and *anti-iso* branched C_{15} fatty acids, both of which are bacterial markers (e.g., Scribe *et al.*, 1991).

5.2.6 Ketones, aliphatic alcohols and methyl esters

Hexadecan-3-one and *n*-hexadecanol were desorbed after the first and second

developments, respectively. Hexadecan-3-one was desorbed at 400 °C without further treatment, while the alcohol was converted to its TMS ether with BSTFA and desorbed intact at 300°C. Some aliphatic alcohols (C_{16}, C_{20}, C_{22}) were detected as TMS ethers in the free sterol band (Fig. 5.5), reflecting the difficulty of accurate band location and thus the need to develop a system which accommodates intact Chromarods. There is current interest in the use of specific alkenones in sediments as paleoclimatic indicators (e.g., Ficken and Farrimond, 1995). Further refinements in the method would be required to allow sensitive analysis of unhydrogenated material, in order not to lose essential information deriving from the C=C double bond position.

Fatty acid methyl esters (FAMEs) occur naturally in the marine environment (Parrish 1988), and Iatroscan TLC-FID detected these as a minor lipid class in 75 m settling particles (Fig. 4.1). After development of unhydrogenated lipids from 75 m settling particles, desorption of the FAMEs at 500°C revealed C_{14}, C_{16}, C_{18} and C_{20} species, with comparable quantities of saturated and monounsaturated C_{16} FAME, and the C_{18} and C_{20} esters being largely monounsaturated. No polyunsaturated species were detected, despite their being a major constituent of the fatty acid profile of these settling particles (Budge and Parrish, 1998). It is possible that they were present but underwent thermal degradation.

5.3 Polar lipid classes

5.3.1 Acetone-mobile polar lipids

1-Monopalmitin (1-monopalmitoyl-*rac*-glycerol), a standard for the AMPL peak in TLC-FID, was desorbed as its bis(trimethylsilyl) ether (peak 2, Fig. 5.6). Peak 1 in the chromatogram is due to the 2-monopalmitin bis(TMS) ether (recognizable by m/z= 218 in its mass spectrum) (Johnson and Holman, 1966). Since the standard as delivered was 99% pure, some of the 1-monopalmitin or its TMS ether isomerized to the 2-form, either during TMS ether formation on the rod or during thermal desorption. Several common silylation reagents reportedly lead to such isomerization when used on monoacylglycerols (Myher and Kuksis, 1974). Nonetheless, the desorption of 1-MAGs in good yield has been demonstrated. In future work, a reagent which does not lead to isomerization, such as pyridine/ hexamethyldisilazane/trimethylchlorosilane, should be used.

Desorption of the AMPL band of hydrogenated lipids from settling particles at 100 m after BSTFA treatment gave a range of bis(TMS) ethers of 1-MAGs, with acyl groups ranging from C_{16} to C_{22} (Fig. 5.6b). No TMS products due to 2-MAGs were observed.

The relative sensitivities of the new method for different constituents (MAGs, glycoglycerolipids, pigments) of this complex band are not known. In this case, pigment was still visible on the Chromarod sections even after their removal from the Pyroprobe following heating, suggesting strong adsorption on the silica. Glycoglycerolipids, also products of photosynthetic algae (Parrish, 1988) were not investigated in this study, although

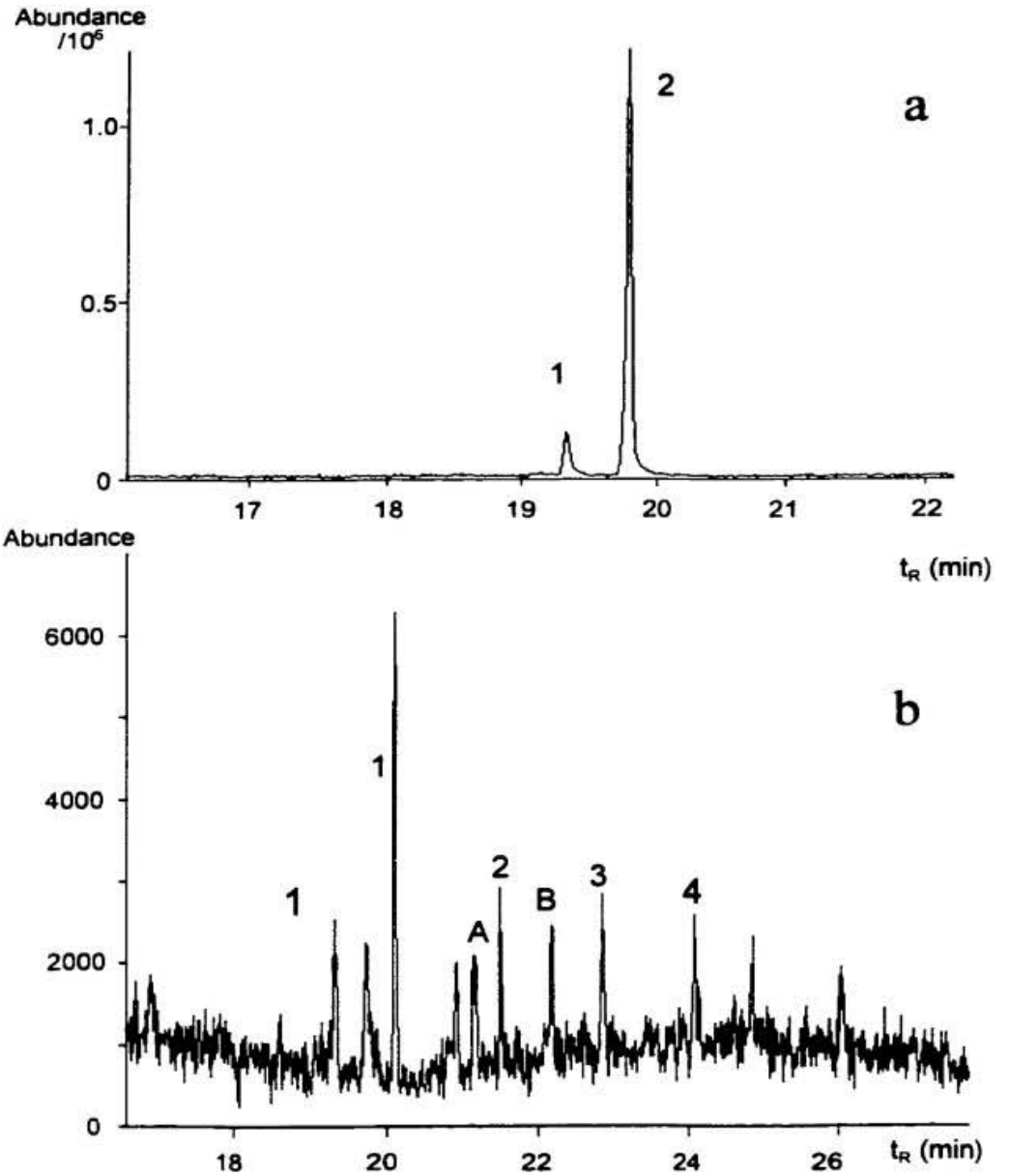


Figure 5.6. Monoacylglycerol desorption after BSTFA treatment; Pyroprobe set at 500 °C. (a) Desorption of a standard (1-monopalmitin). 1, 2-monopalmitin di-TMS ether, due to isomerization; 2, 1-monopalmitin di-TMS ether. (b) AMPL band from 100 m settling particles. Di-TMS ethers of 1-MAGs: 1, C_{16}; 2, C_{18} ; 3, C_{20} ; 4, C_{22}. A, B: unidentified steroids.

they may be amenable to analysis in a manner similar to steryl glucosides (Section 5.3.2). Silylation of the free glycoside hydroxyls may allow desorption of the entire molecule, with subsequent thermal cleavage yielding a species characterized by the fatty acid moiety. To date, the AMPL band in TLC-FID analysis of lipids from settling marine particles has been used to gauge primary productivity, since it contains glycoglycerolipids and photosynthetic pigments attributable to phytoplankton (Parrish, 1998b). However, the demonstrated presence of MAGs suggests that the AMPL band cannot always be unambiguously attributed to algae. MAG may be formed by the breakdown of TAG, DAG or other acyl lipids, and may therefore indicate bacterial activity or other forms of degradation of organic matter.

In addition to MAGs, two compounds in the AMPL band (Fig. 5.6b, peaks A & B) have sterol character (characteristic fragment ions at m/z 215, 147 and 257/255:), raising the intriguing possibility of sterol-containing species in this band. Steryl chlorin esters may be very similar to pigments in their elution behaviour on Chromarods due to their phytopheophorbide *a* moiety (Fig. 1.4) , and may give the sterol moiety on thermal cleavage. Further investigations in this area would be aided by the availability of a well-characterized steryl chlorin ester standard.

5.3.2 Steryl glucosides

A standard containing campesteryl (24α-methylcholesteryl), stigmasteryl (24α-ethylcholest-5,22*E*-diyl) and sitosteryl (24α-ethylcholesteryl) glucosides yielded peaks

attributable to the dehydrated sterols (ions at m/z 382, 394 and 396, respectively) on treatment with both TMAH and BSTFA and, to a far smaller extent, on desorption at 550 °C without any added reagent. Persilylated (Christensen *et al.*, 1982; Kusmierz *et al.*, 1989) and permethylated sugars have been successfully prepared for gas chromatography, and so it is possible that the steryl glucosides were trimethylsilylated or methylated at all the hydroxyl positions on the glucose moeity. However, such a compound is unlikely to elute from a 25-m capillary column with the observed retention time. Furthermore, multiple peaks containing these ions were observed (Fig. 5.7), despite only three sterols being present, and these eluted at the same retention times regardless of the treatment used. While TMAH is known to hydrolyze glycosidic linkages (del Rio *et al.*, 1996), the sterol mass spectra did not indicate methylation at the C-3 oxygen when it was used, and when free campesterol, stigmasterol and sitosterol standards were desorbed with TMAH, peaks due to the sterol methyl ethers were observed at longer retention times than those of the sterol species obtained from steryl glucosides.

Cleavage of the glycosidic linkage, would result in steradienes (Fig. 5.8); this could occur by heat alone or with the assistance of TMAH. The 3,5-dienes (Fig. 5.8, I) would be expected as the major product, being stabilized by conjugation. Some of the 2,5-diene may also be expected (Fig. 5.8, II). Lastly, isomerization of the 3,5-diene may give some of the conjugated 2,4-diene (Fig. 5.8, III). For a given sterol, these three species would have identical molecular ions and very similar fragmentation patterns, but slightly different retention times, leading to multiple GC peaks where only one is expected. The greater

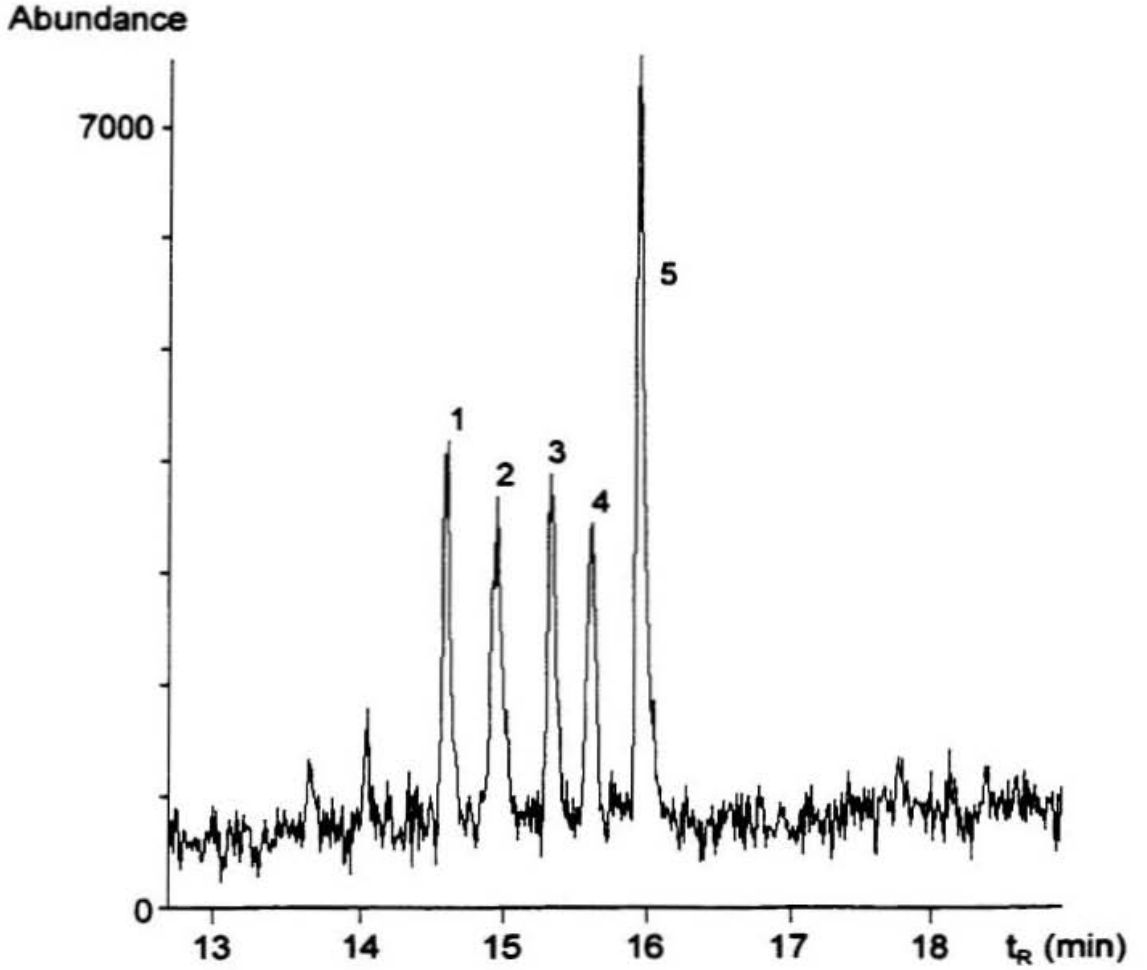


Figure 5.7. Total ion chromatogram resulting from the desorption of campesteryl, stigmasteryl and sitosteryl glucoside (2.3 μg total) from a Chromarod section after BSTFA application. The same result was obtained with TMAH (MeOH) application.

abundance of sterenes observed after treatment with TMAH or BSTFA suggests that thermal or TMAH-assisted cleavage takes place after desorption, since derivatization of the glycoside hydroxyls would decrease the strength of adsorption on the silica surface.

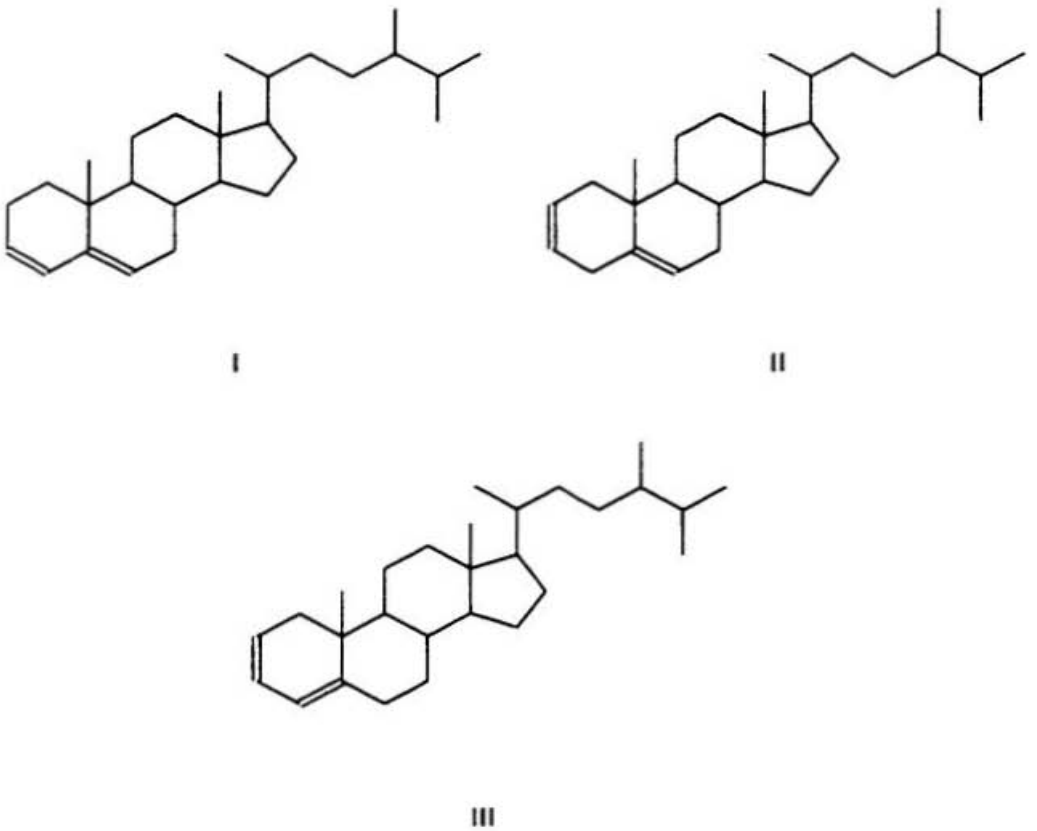


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Figure 5.8. Isomeric steradienes proposed to result from thermal cleavage of campesteryl glucoside.

There is no evidence in steryl glucoside desorption chromatograms of any fragments attributable to the glucose moiety, regardless of the treatment used. Pyrolysis of glucose or saccharides containing it may produce a number of species, including levoglucosan (1,6-anhydroglucose), or its permethylated or persilyilated analogues (Jackman, 1991). Ions characteristic of levoglucosan or glucose, or any of their methylated or silylated analogues, were not observed in the mass spectra of any GC peaks from steryl glucoside desorption. Lastly, pyrolysis of glucose can produce low molecular mass species such as 2-furfural and hydroxymethylfurfural (Irwin, 1982). If these species were produced, they would have eluted from the column during the early, fast temperature ramp and therefore would not have been detected due to the detector solvent delay. Alternatively, the polar, porous silica surface may simply have retained the glucose moiety or its products. This is conceivable since other polar compounds, such as pigments, were not removed by pyrolysis to an appreciable extent. However, this is inconsistent with desorption being easier after silylation or methylation has inactivated the glucose hydroxyl groups.

Co-spotting of steryl glucoside and phosphatidylcholine standards, followed by the third development, showed that they elute in the same area of the Chromarod, the former eluting approximately 1 cm ahead of the latter without full baseline separation (Fig. 5.9). This suggests caution in the use of TLC-FID to monitor total phospholipids, since at least one other class of substances may elute in the same region of the chromatogram.

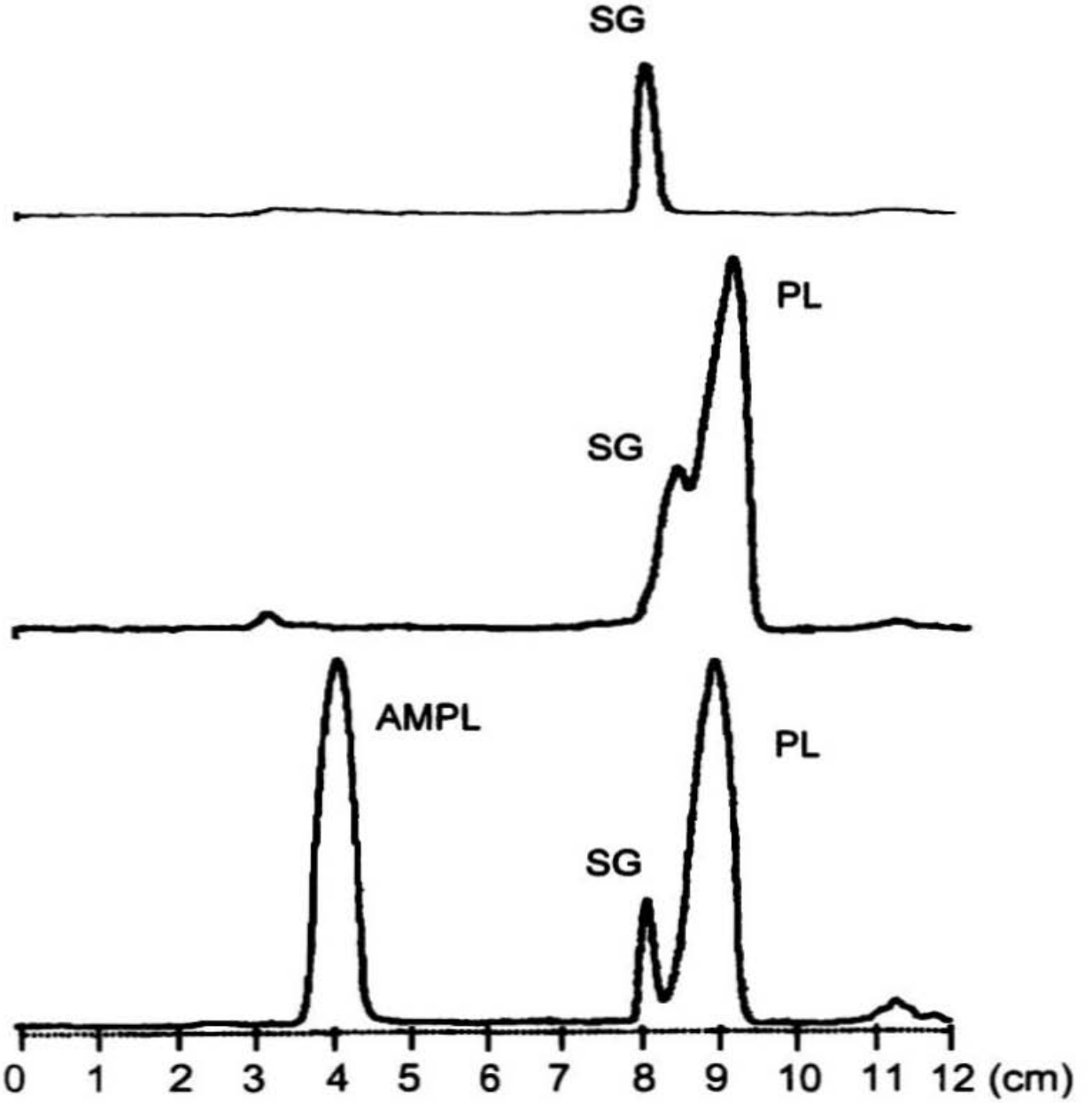

Figure 5.9. Elution of steryl glucoside (SG) standard in the third development (Section 4.4). The upper two chromatograms were subjected to the third development only; the lower one, to all three developments. PL standard: dipalmitoylphosphatidylcholine; AMPL standard: 1-monopalmitin.

5.3.3 Phospholipids

Phospholipids could not be desorbed without further treatment, likely due to their very polar head groups. Thermochemolysis with TMAH yielded FAMEs from the phospholipid acyl groups, with the treatment of dipalmitoylphospatidylcholine giving an abundant C_{16} FAME peak. To date only phosphatidylcholine and ethanolamine standards have been investigated, and so it is not known whether the method discriminates between various phospholipid subclasses. The differing structures of their phosphatidyl head-groups influence the strength of their absorption to silica, and so this is an important topic for further study.

The phospholipid band of hydrogenated settling particulate lipids (100 m) desorbed in this way showed a maximum at C_{16} in its carbon number distribution, with roughly equal amounts of C_{14}, C_{18}, C_{20} and C_{22} present at approximately one-third this level. Very low levels of C_{14}, C_{16} and C_{18} FAMEs were detected in phospholipids at 75 m.

Any steryl glycosides present in this band would presumably have been detected as the corresponding sterenes, as occurred with steryl glucoside standards. Since no sterenes were observed, steryl glycosides would be present only at a concentration below the 2.3 μg detection limit for these compounds (the lowest level observed with TMAH treatment), if at all, although this would still constitute 27 % of the 8.6 μg phospholipid peak. However, the absence of any small peak close to the phospholipid peak in the TLC-FID chromatograms (e.g., Fig. 4.1) suggests that no steryl glycosides are present.

5.4 Pyroprobe interface: resolution and reproducibility considerations

A number of factors may contribute to the rather broad peaks and tailing apparent in several of the chromatograms of desorbed lipids. During desorption, some back diffusion of the analyte vapour may take place in the dead volume surrounding the platinum coil before the material is swept onto the column. Desorbed analytes are introduced into the column as a solventless vapour and travel down the section of column threaded into the heated interface before encountering the cool oven, which does not allow solvent re-condensation and refocusing on the column head by the solvent effect (Grob and Grob, 1978) although cold trapping can still occur. Furthermore, it is not known whether desorption/pyrolysis is instantaneous or whether it occurs during the entire length of the 20 s heating pulse. Lastly, the interior of the interface is stainless steel, rather than deactivated glass or quartz like a normal injector port lining which is designed to minimize analyte adsorption and thermal decomposition. While contact with this surface by the desorbed sample would be minimal, some adsorption may take place, which would contribute to peak tailing and possibly to decomposition.

Currently, this method is useful only as a qualitative or semi-quantitative identification tool for lipid molecular species. The need to use the split mode (due to a back-pressure regulated injector port on the HP-5890 GC instrument) contributed to problems with sensitivity and poor reproducibility (Table 5.1). Micrograms of material are normally available in a Chromarod TLC band. This amount should be ample for quantitative molecular species analysis of lipids, being 2- 3 orders of magnitude greater than the amount of material

normally required for GC. Much of the desorbed material may have been lost through the split vent. The split injection mode is also poorly suited to quantitation, and this may be exacerbated by the fast He flows used at the start of each run to sweep material onto the GC column, which makes it difficult to establish a stable split ratio.

Table 5.1. Reproducibility of desorption/pyrolysis from Chromarod fragments, as illustrated for steryl and wax esters. 1.2 μg cholesteryl arachidate, 1 μg each of 16:0/16:0 and 18:0/16:0 wax esters. Peak 1= 3,5-cholestadiene; peak 2= 16:0/16:0; peak 3= 18:0/16:0

Peak	Peak ratios					Mean	% RSD
	1	2	3	4	5		
peak 1/ peak 2	0.247	0.359	0.15	0.128	0.914	0.36	90
peak 2/ peak 3	3.05	4.5	2.46	2.43	3.71	3.23	27
peak 1/ peak 3	0.754	1.62	0.366	0.311	3.39	1.29	100

5.5 Anticipated directions in the development of TLC-pyrolysis-GC/MS

A lot of material in each band may be staying adsorbed to the rod coating during pyrolysis or desorption. Even at 800-900 °C, Mukherjee (1974) observed that some lipids were not completely removed from a SiO_2 layer by pyrolysis. In this work, some analytes have been converted to forms more easily desorbed from silica or cleaved; however, the use of reagents which reversibly alter the silica surface to facilitate desorption may also be feasible. Ramaley *et al.* (1985) obtained greater sensitivity in the laser desorption and

detection of material from TLC plates after silanization of the SiO_2 stationary phase.

The current approach does negate one of the major advantages of Chromarod TLC, namely that the rods can be re-used 100 times or more (Parrish 1987). An eventual future goal in the development of this technique is to modify the Pyroprobe interface to accommodate an intact rod and desorb each band by sequentially heating in different places along its length, thus preserving the rod for repeated use. A further advantage of this would be the certainty of desorbing an entire band. Currently, lipid class bands are cut from a rod after their locations are determined based on the scanning of adjacent, replicate rods. Where band locations are not identical on all rods, this can lead to the loss of one or both edges of the band of interest or contamination from adjacent bands (e.g., Section 5.2.2).

6. Conclusions

The use of sterols as source biomarkers in a cold-ocean ecosystem has been demonstrated. The sterol composition of Trinity Bay plankton net tow material was predominated by the C_{27} sterols cholesterol, desmosterol, and *trans*-22-dehydrocholesterol, and the C_{28} sterols 24-methylenecholesterol and brassicasterol, all suggestive of marine (especially diatom) input. The sterol composition of settling particles and especially sediments was more complex than that of plankton. The terrestrial sterols 24-ethylcholesterol and 24-methylcholesterol and their stanol analogues were prevalent in the sediments (<28% for 24-ethylcholesterol alone). Sterol-based source apportionation indicates that at inshore sites <61 % of the sterols are terrestrial, and even at off-shore St-7, <30% of the sedimentary sterols are terrestrial. This suggests that terrestrial materials, including those introduced by human activity, may become widespread in the marine ecosystem. While no typical dinoflagellate sterols were detected in the plankton or settling particles, dinoflagellates appear to be an important contributor to sedimentary organic matter, as dinosterol and dehydrodinosterol occurred in all sediments.

The fecal sterol coprostanol was a minor component of the H-1 and H-9 sedimentary sterols and was not detected in any other samples. Its low abundance and ratio to cholesterol suggest that sewage input is minimal, is readily dispersed or degraded, or is deposited only in the immediate vicinity of sewage outfalls.

To address the limitations of Iatroscan TLC-FID, the method used for determination

of total free sterols, a new technique (TLC-Pyrolysis-GC/MS) was developed. Ten neutral and polar lipid classes have been desorbed from silica-coated Chromarods for direct GC/MS analysis. Steryl esters, wax esters, hydrocarbons, ketones and fatty acid methyl esters were desorbed without pre-treatment. Free sterols, monoacylglycerols, aliphatic alcohols, free fatty acids and steryl glucosides required *in situ* conversion to TMS derivatives on the rod before desorption. Triacylglycerols and phospholipids were converted to fatty acid methyl esters by thermochemolysis with TMAH. The composition of separated lipid bands from settling particles has been elucidated qualitatively or semi-quantitatively. This was particularly valuable for bands containing more than one lipid class (steryl ester/wax ester, acetone mobile polar lipid). The wax ester/steryl ester band contained no more than 8% steryl esters, suggesting that steryl esters are a minor component of this band but may be an important fraction of the total sterol pool. The composition of the steryl ester band (only C_{27} detected) agrees with that of the free sterol band, which in turn matches the carbon number distribution found by saponification, extraction and trimethylsilylation GC analysis for sterols.

In six individual acyl lipid classes, C_{14}-C_{22} fatty acids were detected, with C_{16} acids predominant in all but wax esters. C_{16}-C_{22} monoacylglycerols were identified in the complex acetone-mobile polar lipid band. Moreover, the fatty acid chain lengths present in six individual acyl lipid classes have been revealed, information unobtainable by transmethylation of the TLE. Thus, the method greatly extends the scope of Iatroscan TLC-

FID. Anticipated improvements in the method would include modifying the Pyroprobe interface to accommodate an intact Chromarod, thereby preserving the latter for repeated use.

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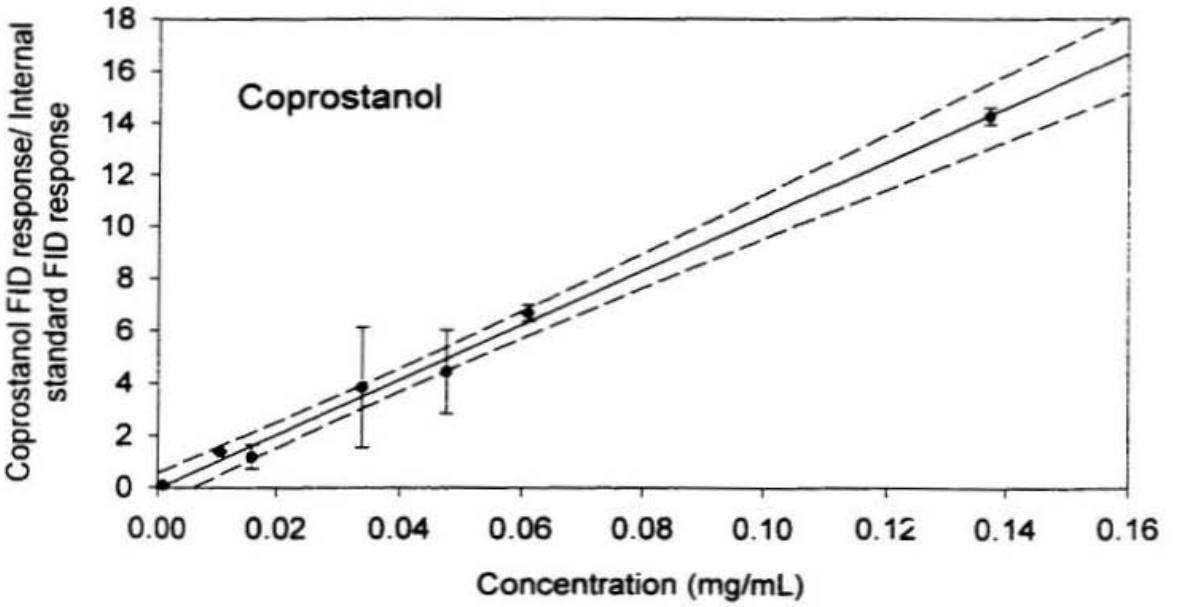
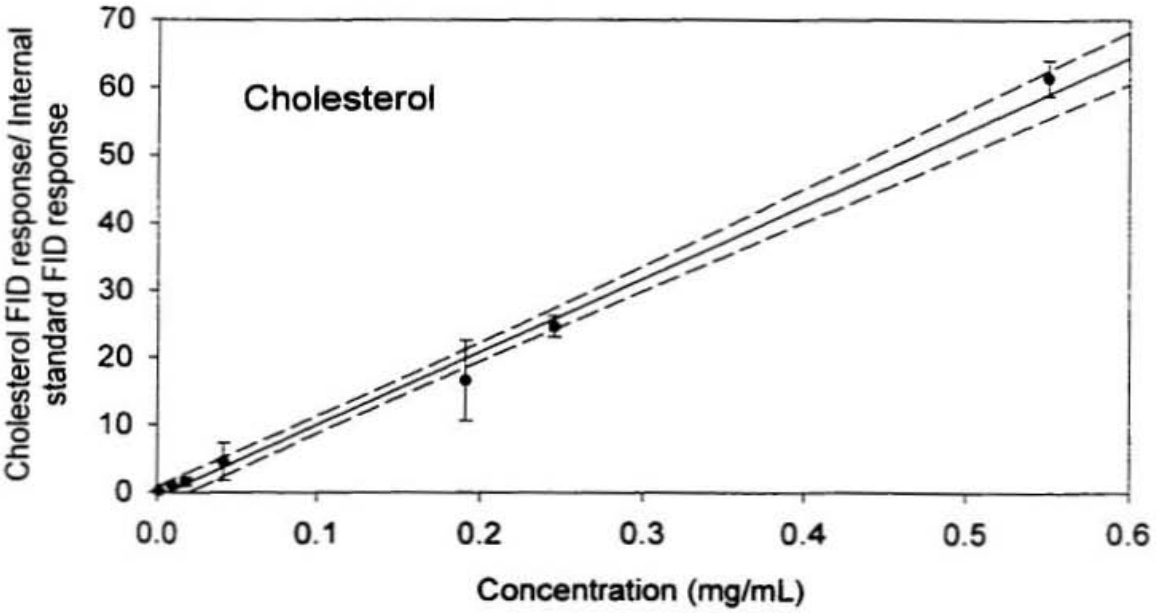
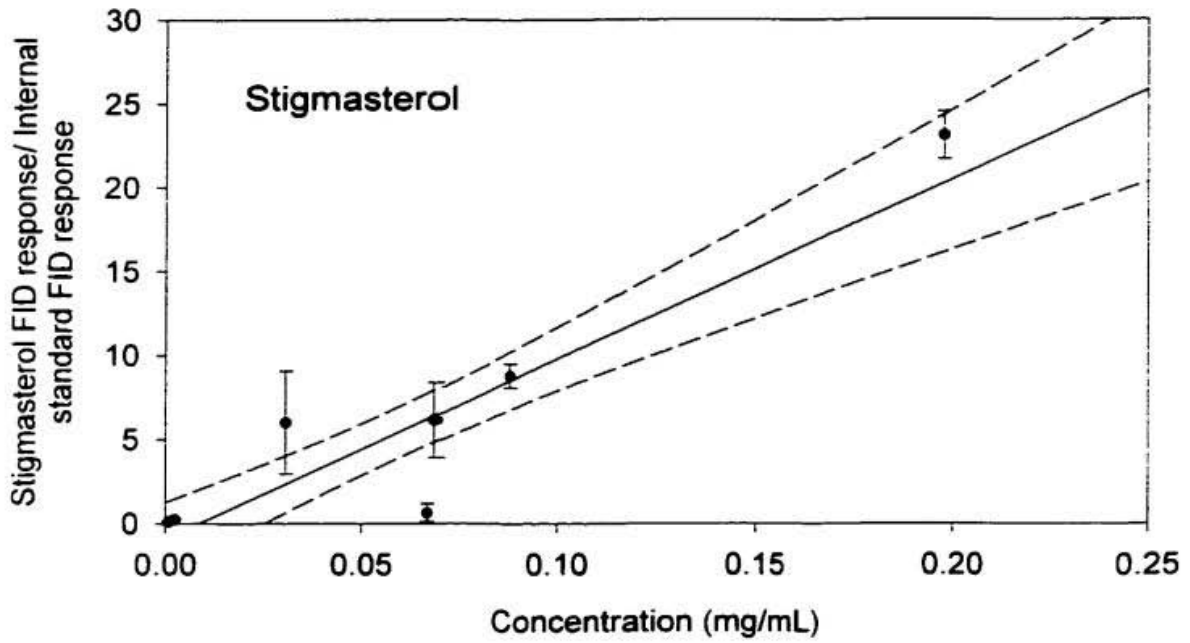
Appendix A. Calibration curves for coprostanol, cholesterol and stigmasterol. For each plot, n=21; points are means ± SD; regressions were performed using the individual data. Dashed lines represent 95 % confidence intervals.





Appendix A. (Continued)



Appendix B. Proportions of individual sterols (% of total) in sediment core horizons. - = none detected.

H-1 Depth (cm)	0-2	2-4	6-8	10-12	14-16	18-20	20-22	22-24	26-28	Mean ± SD
24-nordehydrocholesterol	6.2	4.4	4.1	5.2	8.7	4.7	7.2	6.8	1.6	5.4 ± 2.1
coprostanol	-	1.3	1.6	2.2	-	-	-	-	2.1	0.8 ± 1.0
*epi*coprostanol	2.1	1.0	1.9	2.3	5.7	5.6	2.7	6.5	1.8	3.3 ± 2.1
occelasterol	-	-	-	-	1.1	-	-	1.8	3.6	0.7 ± 1.3
trans-22-dehydrocholesterol	0.61	1.0	-	2.0	2.9	1.9	-	1.5	3.5	1.5 ± 1.2
trans-22-dehydrocholestanol	1.3	-	-	-	-	0.21	0.87	3.1	-	0.6 ± 1.0
cholesterol	3.8	3.1	3.3	4.0	5.1	4.6	4.7	3.9	5.5	4.2 ± 0.8
cholestanol	2.5	2.7	2.3	3.1	2.9	2.8	1.8	2.4	3.7	2.7 ± 0.5
(*epi*)brassicasterol	6.2	2.4	2.6	3.1	2.9	3.2	1.9	3.2	5.1	3.4 ± 1.4
(*epi*)brassicastanol	2.0	0.24	2.0	2.6	1.8	2.3	1.0	1.0	2.9	1.8 ± 0.9
24-methylenecholesterol	1.9	1.4	1.4	2.2	2.0	1.4	1.8	4.1	2.4	2.1 ± 0.8
24-methylcholesterol	6.5	8.5	6.1	4.6	10	4.8	7.8	6.3	5.3	6.6 ± 1.8
24-methylcholestanol	1.8	1.7	4.3	5.3	1.6	3.1	2.8	1.4	6.7	3.2 ± 1.9
ethylcoprostanol	2.8	-	-	-	-	-	5.8	-	-	1.0 ± 2.0
dimethyldehydrocholestanol	2.0	3.4	-	-	-	-	-	-	-	0.6 ± 1.2
ethylcholesta-5,22-dienol	1.8	0.09	2.7	2.9	2.6	1.8	2.7	3.2	2.9	2.3 ± 1.0
ethylcholest-22-enol	-	0.22	1.6	2.8	2.6	2.8	2.8	3.5	3.2	2.2 ±1.3
24-ethylcholesterol	23	25	28	27	28	26	30	26	27	26 ± 2
24-ethylcholestanol	8.3	8.5	7.7	7.4	7.9	7.0	9.5	7.1	8.0	7.9 ± 0.7
dehydrodinosterol	1.2	1.3	3.4	-	-	3.7	-	2.6	-	1.3 ± 1.5
dinosterol	3.7	6.0	7.7	5.2	3.8	4.7	2.8	3.5	4.0	4.6 ± 1.5
C_{30} steratrienol	18	23	14	6.2	7.1	15	14	9.0	4.6	1.2 ± 6.2
Unidentified sterols	3.9	4.8	5.7	12	3.7	4.0	0	3.4	6.7	4.9 ± 3.2

Appendix B. (Continued)

H-9 Depth (cm)	0-2	2-4	6-8	10-12	12-14	16-18	18-20	22-24	26-28	28-30	Mean ± SD
24-nordehydrocholesterol	7.8	4.0	7.0	3.5	5.6	4.6	6.3	4.7	3.5	4.4	5.1 ± 1.5
24-nordehydrocholestanol	1.7	1.8	-	3.0	1.4	2.0	1.2	3.2	2.7	1.4	1.8 ± 1.0
coprostanol	-	2.5	3.9	1.0	4.4	1.6	1.6	2.9	2.9	-	2.1 ± 1.5
*epi*coprostanol	2.0	1.4	3.7	4.1	3.8	2.2	2.1	2.1	3.6	3.3	2.8 ± 1.0
occelasterol	-	2.0	1.0	1.4	1.5	0.89	0.66	1.0	1.8	1.6	1.2 ± 0.6
trans-22-dehydrocholesterol	0.41	0.83	0.72	3.1	1.2	0.91	0.97	0.66	1.1	-	1.0 ± 0.8
trans-22-dehydrocholestanol	-	2.0	2.7	-	1.6	2.1	2.0	2.0	2.3	-	1.5 ± 1.1
cholesterol	7.5	6.3	5.7	4.3	6.3	4.4	4.5	4.2	5.0	6.5	5.5 ± 1.2
cholestanol	4.3	4.8	4.6	5.3	4.2	4.7	4.4	4.0	4.7	5.4	4.6 ± 0.4
(*epi*)brassicasterol	4.3	6.1	4.6	2.6	3.7	3.5	3.5	3.5	3.1	3.5	3.8 ± 1.0
(*epi*)brassicastanol	2.0	2.6	3.2	1.4	2.0	2.4	2.4	2.4	2.8	1.7	2.3 ± 0.5
24-methylenecholesterol	3.8	3.7	4.2	1.5	2.2	3.0	2.7	3.1	3.1	4.9	3.2 ± 1.0
24-methylcholesterol	7.6	6.9	7.6	4.2	7.6	8.8	8.8	9.1	6.8	8.4	7.6 ± 1.4
4-methylcholestanol	-	-	-	-	0.59	1.0	1.0	-	-	-	0.3 ± 0.4
24-methylcholestanol	3.1	2.4	3.3	1.8	2.9	3.6	3.5	3.7	3.8	4.7	3.3 ± 0.8
ethylcholesta-5,22-dienol	3.0	4.1	2.9	2.9	1.5	2.4	1.9	2.3	2.3	1.4	2.5 ± 0.8
24-ethylcholesterol	17	14	15	2.6	16	18	15	16	16	19	15 ± 5
24-ethylcholestanol	5.8	8.4	5.1	12	6.4	9.6	5.5	7.7	5.5	8.7	7.4 ± 2.1
fucosterol	5.5	-	3.6	19	4.5	0.55	3.4	2.4	3.5	5.7	4.8 ± 5.4
dehydrodinosterol	4.9	4.4	4.2	7.4	3.5	5.0	4.4	4.0	4.0	4.4	4.6 ± 1.1
dinosterol	1.2	4.4	2.7	5.0	1.1	1.4	2.0	1.9	1.8	0.88	2.2 ± 1.4
C_{30} steratrienol	4.0	5.0	2.5	1.5	3.0	3.6	3.3	4.8	1.5	3.9	3.3 ± 1.2
Unidentified sterols	14	12	12	12	15	14	19	14	19	10	14 ± 3

Appendix B. (Continued.)

St-7	Depth (cm)	2-4	6-8	10-12	14-16	18-20	22-24	26-28	30-32	Mean ± SD
24-nordehydrocholesterol		3.1	2.8	3.0	3.4	3.0	3.2	3.1	2.8	3.1 ± 0.2
24-nordehydrocholestanol		3.5	3.1	1.9	2.2	2.3	2.1	5.2	2.9	2.9 ± 1.1
*epi*coprostanol		3.4	4.0	2.0	2.5	2.6	2.0	5.2	1.6	2.9 ± 1.2
occelasterol		0.98	1.6	1.2	0.93	0.97	1.0	2.9	0.94	1.3 ± 0.7
trans-22-dehydrocholesterol		0.80	2.9	2.5	0.15	2.8	3.0	2.9	3.3	2.3 ± 1.2
trans-22-dehydrocholestanol		2.4	4.4	6.3	2.9	5.4	5.3	4.9	3.7	4.4 ± 1.3
cholesterol		5.7	4.7	6.1	7.2	7.2	7.7	6.7	8.1	6.7 ± 1.1
cholestanol		6.9	8.0	7.4	8.5	7.5	7.1	8.7	7.7	7.7 ± 0.6
(*epi*)brassicasterol		4.4	5.0	4.8	6.4	6.0	4.3	3.8	5.4	5.0 ± 0.9
(*epi*)brassicasterol		3.4	3.5	3.4	3.8	3.1	3.2	2.1	3.0	3.2 ± 0.5
24-methylenecholesterol		3.8	4.4	4.5	6.0	4.9	4.3	2.8	3.6	4.3 ± 0.9
24-methylcholesterol		15	12	12	9.9	10	9.1	6.4	8.0	10 ± 3
24-methylcholestanol		4.1	3.5	3.6	3.7	3.5	2.9	1.5	2.2	3.1 ± 0.9
ethylcholesta-5,22-dienol		1.6	2.5	1.6	2.0	1.8	1.7	2.0	2.1	1.9 ± 0.3
24-ethylcholesterol		7.2	7.0	7.5	5.4	7.1	8.6	15	11	8.7 ± 3.2
24-ethylcholestanol		6.2	7.5	6.7	6.9	6.7	6.5	5.3	5.7	6.4 ± 0.7
fucosterol		3.5	2.8	3.5	5.1	3.1	4.0	2.3	4.6	3.6 ± 0.9
dehydrodinosterol		3.8	4.0	5.2	7.0	3.7	7.1	4.6	3.4	4.9 ± 1.5
dinosterol		1.8	1.7	1.5	1.4	1.5	2.4	1.5	2.8	1.8 ± 0.5
Unidentified sterols		18	15	15	14	16	15	13	17	15 ± 2