

Lipid and Phenolic Biomarkers in Marine Ecosystems: Analysis and Applications

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Biomarkers are compounds or groups of compounds that can be used as signatures of individual organisms or groups of organisms, or of certain environmental processes. Lipid and phenolic biomarkers can be used to assess the health of an ecosystem and the degree to which it has been influenced by terrestrial and anthropogenic inputs. Lipid classes and fatty acids can be used to determine production of marine biogenic material of dietary value to pelagic and benthic organisms. Polycyclic aromatic hydrocarbons and 5 β -stanols such as coprostanol can be used to determine pollutant loading from oil spillage or sewage and the phenanthrene/methylphenanthrene ratio can be used specifically as an indicator of wood burning. *N*-alkanes and thermochemolysis products in cores can show the sensitivity of sediments to changes in land use patterns near the land margin. The relationship between marine and terrestrially derived products in sediment cores can be used to indicate the degree to which land use changes have impacted the pattern of marine biogenic productivity in the area. Stable isotope and multivariate analyses are particularly useful for biomarker validation.

Key words: Molecular signatures, Stable isotopes, Chemometrics.

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Introduction

Molecular biomarkers are easily determined compounds that tell us about the history of a sample. They can be signatures of the condition of a sample, they can tell us about past events and even about the future when certain compounds are used as early warning signals. Both molecular and isotopic analyses of biomarkers have been extensively used in geochemical studies [e.g. 1, 2] but there is now increasing interest in their use in ecological studies. Indeed, further biomarker studies of modern environments will greatly aid source identification in sediments [e.g. 3].

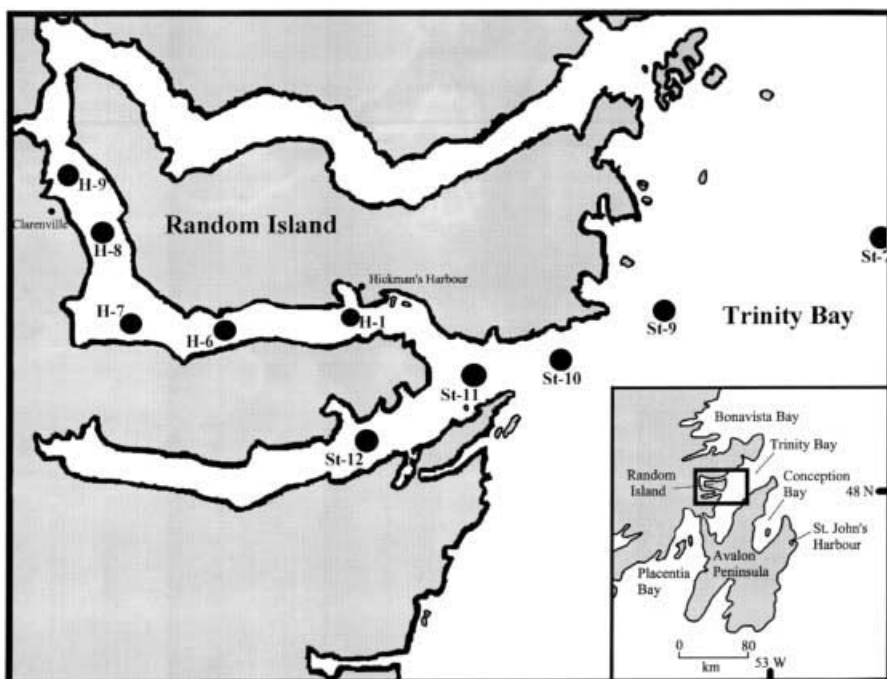


Fig. 1. Station locations in Trinity Bay and around Random Island. Trinity Bay is a large fjord-like bay which historically had an abundance of spawning cod during spring. It is about 100 km long and 30 km wide, with a maximum depth of about 590 m and is representative of several cold ocean bays around Newfoundland

A marine biomarker can be a DNA fragment or an enzyme that shows us that a fish or an invertebrate has been exposed to a xenobiotic [e.g. 4–7], but our focus here will be on smaller molecules that are determined using standard chromatographic techniques. Lipids are one such group that are receiving increasing amounts of attention in ecological [8] and biogeochemical [9, 10] studies. Less well known is the biomarker potential of phenolic compounds determined by pyrolysis gas chromatography. In this chapter we will compare and contrast the use of these two types of biomarkers using research we undertook in Trinity Bay, Newfoundland as a case study (Fig. 1). The use of biomarkers to investigate the marine ecosystem in the cold waters off the coast of Newfoundland is new and timely given current problems with the ground fishery [11, 12] and the increasing interest in aquaculture and oil field development in the area. This is the first study to compare different lipids and phenolics in plankton, settling particulate material and sediments in a cold fjord-like environment. Polycyclic aromatic hydrocarbons and fecal sterol biomarkers were used to evaluate pollutant loading. In addition, the PAH distribution patterns were determined in order to fingerprint anthropogenic sources as being petrogenic or pyrolytic. Total lipids, lipid classes, polyunsaturated fatty acids and sterols were used to examine marine inputs, as were total fatty acids derived from thermochemolytic analyses. The $\Sigma nC_{26}-nC_{35}/\Sigma nC_{16}-nC_{25}$ *n*-alkane ratio and phenolic compounds were used for terrestrial plant inputs to the marine ecosystem. Finally, 3,4-dimethoxybenzoic acid methyl ester was used as a specific marker for wood inputs.

2

Lipid Classes

Lipids are carbon-rich compounds with a very high energy value, making them important fuels in marine ecosystems. Marine lipids are usually extracted using some kind of simplification of the Folch et al. [13] procedure in which the sample is ground in chloroform and methanol (2:1). Such an extract may contain as many as 16 different subclasses of both biogenic and anthropogenic origin [14]. The heterogeneous nature of lipids means that much information can be gained by determining individual classes and in the process, lipids can be separated from non-lipid contaminants.

Lipid classes can be separated by thin layer chromatography on silica gel coated Chromarods [15] or plates [16, 17]. Both rod and plate TLC are amenable to quantitation: plates can be scanned in a densitometer, while Chromarods can be passed through the flame ionization detector of an Iatroscan. An advantage of the Chromarod-Iatroscan TLC/FID system is the partial scanning facility that permits extensive analysis of a single sample on a single rod. By separating out all the lipid classes in this way, much greater confidence in the identities of individual peaks is obtained and any non-lipid material remains at the origin. In addition, the Chromarod-Iatroscan procedure is sensitive, with a detection limit of around 50 ng, and it has been successfully used in an intercalibration exercise between laboratories in different countries [18]

Marine samples with high proportions of polyunsaturated fatty acids may present problems with identification and quantification by TLC/FID. With

Chromarod separations, peak splitting may occasionally be observed in the wax ester or triacylglycerol region of chromatograms. This is due to the presence of molecular species with widely differing degrees of unsaturation [19], and it could lead to misidentification. The second part of a split wax ester peak could be mistaken for a fatty acid methyl ester and the second part of a split triacylglycerol peak could be mistaken for a free fatty acid. There may also be a problem if Iatroscan calibration has been done with saturated standards. This could cause an 18–70% underestimation of the amount of a lipid class present [20, 21] because unsaturated lipids give a lower detector response and because of the broadening of Chromarod bands containing significant proportions of polyunsaturated molecular species. Band broadening also results in lower responses [22, 23]. However, the difference in absolute response due to unsaturation is quite small [19] and the effect of band broadening can be counteracted by exposing the band to multiple developments in schemes that involve solvent focusing, double developments, or partial scanning and redevelopment. The fact that Iatroscan values for aquatic samples are routinely 80–95% of those obtained by gravimetry and other methods [18, 24–28] attests to the general applicability of saturated standards in calibrations.

Another approach to obtaining synoptic marine lipid class data is by short column gas chromatography [29]. In terms of the information provided, this profiling method is located between detailed fatty acid analyses and the class analyses provided by the Chromarod-Iatroscan system. It groups compounds according to carbon number within each class. By summing the groups of molecular species within each class, total class amounts can be obtained. This method is readily automated and has been successfully applied to a wide range of marine samples [29].

In marine ecosystem studies, two lipid classes that are of particular interest are the triacylglycerols and phospholipids which are biochemically related. They both possess a glycerol backbone to which 2 or 3 fatty acids are esterified and they also share a common precursor. Triacylglycerols are a very important energy storage substance and have been used as a condition index for marine fauna [30]. In our Trinity Bay study (Fig. 1) we found that in spring, input rates of total lipids increased to a level typical of a highly productive oceanic upwelling region [31]. At this time, the highest flux of triacylglycerol was observed in the lipid material falling through the water column (Fig. 2), indicating storage of energy in bloom organisms and its transfer to the benthos. The triacylglycerol fluxes measured at all 3 depths in late spring, together with some of those observed during summer, summer/fall, and early spring in Trinity Bay were higher than those reported for various oceanic regions, including the Peru upwelling region [31]. The high triacylglycerol fluxes in late spring are undoubtedly related to low nutrient concentrations: diatoms increase triacylglycerol synthesis when nitrate or silicate supply is low [32–34]. Triacylglycerols, together sometimes with steryl esters and free fatty acids, are also important in determining PCB concentrations in marine biota [35].

Phospholipids are essential components of membranes where they share a structural function with sterols. Phospholipids can be used to indicate freshly biosynthesized material [36] and individual ones may be used to distinguish

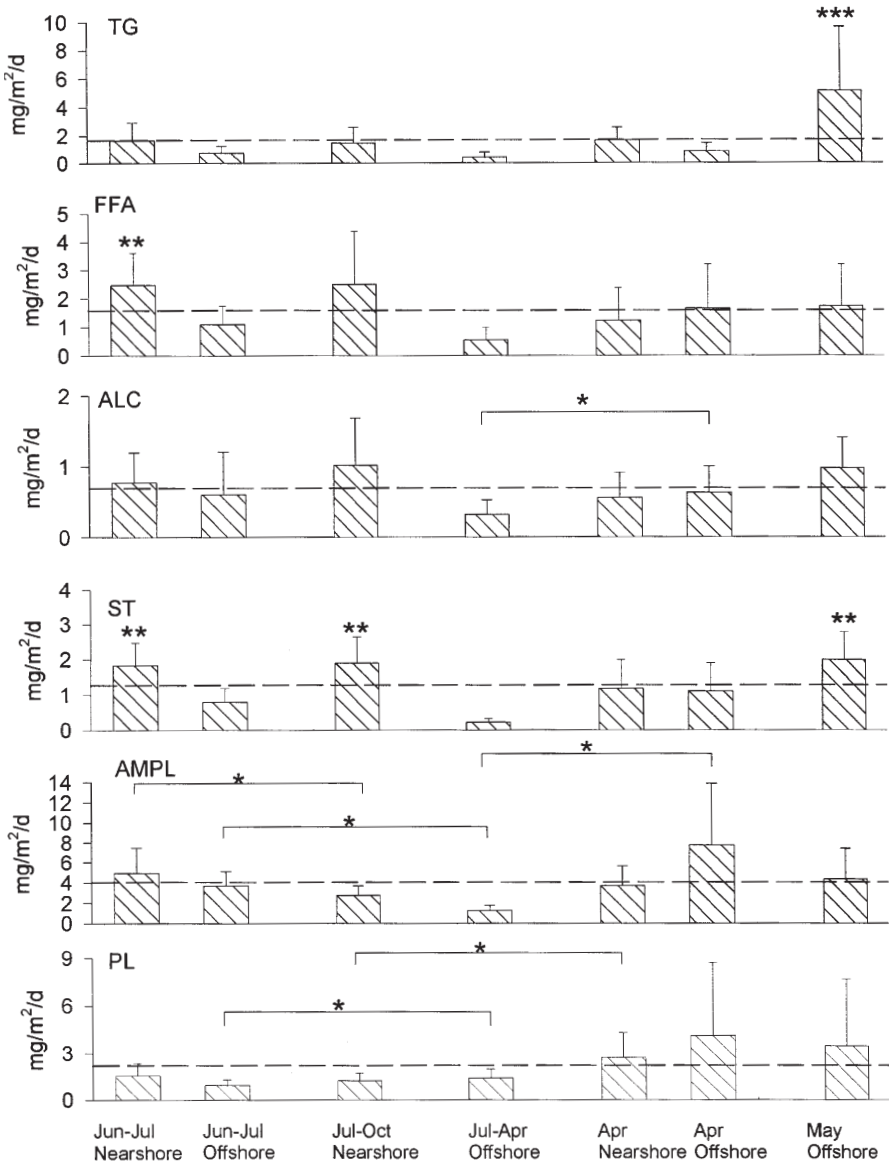


Fig. 2. Fluxes of particulate matter through the water column in Trinity Bay, Newfoundland. Rates are the averages of traps at 3 depths (50, 75, 100 m) for lipid class inputs. The error bars indicate one standard deviation above the mean; the dashed line is the annual mean flux. TG: triacylglycerol, FFA: free fatty acid, ALC: alcohol, ST: sterol, AMPL: acetone-mobile polar lipids, PL: phospholipid. *** $p < 0.05$: Significantly higher than the annual mean flux as well as all other fluxes. ** $p < 0.05$: Significantly higher than the annual mean flux. * $p < 0.03$: Significantly different fluxes in different seasons

bacteria and phytoplankton [37, 38]. In Trinity Bay, the highest fluxes of phospholipids occurred in spring (Fig. 2) indicating massive transport of membrane material through the water column. The flux of acetone-mobile polar lipids was also highest at this time. This class is a mixture of pigments and chloroplast-associated glycolipids and thus strongly indicates plant material transiting the water column. The lowest fluxes of almost all lipid classes were found in the fall/winter samples.

Ketones are a class of lipids that were rarely seen in plankton and sediment trap samples from Trinity Bay, although there was a significant level in sediments. This suggests the possibility of their use as an internal standard in lipid analyses of water column samples. However, they are present in some prymnesiophytes and, in fact, there is much interest in their use as paleotemperature indicators [e.g. 39, 40], although methyl and ethyl ketone concentrations respond to nitrogen limitation as well [41]. Their rarity combined with the fact that they may comprise as much as a quarter of the lipids of some species [42], however, suggests that as a class, ketones could be excellent water column markers in some areas.

Lipid breakdown can be assessed from the free fatty acid content [14, 36, 43] or the free fatty acid plus alcohol content [44]. The proportions of free fatty acids were at their lowest during the spring bloom in Trinity Bay with values as low as 5%, reflecting a relative lack of acyl lipid degradation at that time. Fluxes of free fatty acids and free aliphatic alcohols, were highest in the summer/fall samples. The high alcohol flux suggests degradation of zooplankton-derived wax esters and microalgal chlorophylls was greatest then. However, free fatty acid levels and perhaps free alcohol levels may be easily overestimated. Recent papers suggest free fatty acid levels in diatoms should really be close to zero, and that problems are encountered at the sample collection and extraction stage [45, 46]. The use of boiling water to deactivate lipolytic enzymes is to be recommended.

An index to indicate the degree of breakdown has been suggested by Weeks et al. [47] which they term the hydrolysis index (HI). It is defined as:

$$HI = \frac{(\text{free fatty acids} + \text{alcohols})}{(\text{total non-polar acyl lipids} + \text{products})} \times 100$$

An alternative lipolysis index based on that of Weeks et al. [47] has also been proposed [44]:

$$LI (\%) = \frac{(\text{free fatty acids} + \text{free alcohols})}{(\text{total acyl lipids} + \text{free alcohols})} \times 100$$

This lipolysis index is usually strongly correlated with that of Weeks et al. However, it differs from theirs in that it takes into account all sources of hydrolysis products, polar and non-polar, since only hydrocarbons and sterols are missing in the denominator. In our Trinity Bay study, the lipolytic breakdown indices of net-tow samples were the same as in the sediment trap material suggesting plankton lipids were well preserved in traps.

Thus, in addition to overall caloric content, lipid class data can provide information about the condition of a sample in terms of age of material or nutrient limitation of algae. However, lipid class information is particularly valuable when used in conjunction with determinations of individual compounds.

3 Fatty Acids

Acyl lipids are almost always the major contributors to a marine lipid extract. The fatty acid moieties are often determined individually as fatty acid methyl esters (FAME) which are commonly produced from lipid extracts by transesterification using one of several techniques. We have found that a simple and rapid method employing 10% boron trifluoride in methanol at 85 °C [48] produces equivalent or superior yields to other acid-catalyzed techniques with no evidence of polyunsaturated fatty acid (PUFA) loss or artifact formation. FAMES are most commonly analyzed using a gas chromatograph (GC) equipped with flame ionization detection. The use of a mass spectral detector is invaluable in determining the structure of unusual fatty acids. A polar GC column, such as one coated with a polyethylene glycol [49], is necessary for adequate separation of isomers and a gas-line oxygen scrubber is essential [50]. In this way 30–35 fatty acids can be routinely separated. They can be named using a convenient shorthand notation of the form A:B ω X, where A represents the number of carbon atoms, B gives the number of double bonds and X gives the position of the double bond closest to the terminal methyl group. With this naming system, all double bonds are assumed to be methylene-interrupted and *cis* in configuration.

Using fatty acid biomarkers, general phytoplankton sources in marine samples may be determined. The ratios of 16:1/16:0 and the sum of all fatty acids having 16 carbon atoms to the sum of all fatty acids having 18 carbon atoms ($\Sigma C_{16}/\Sigma C_{18}$) were originally proposed by Claustre et al. [51] as diatom markers. In conjunction with this, Bodennec et al. [52] suggested that values of 16:1/16:0 greater than 1.6 could be interpreted as signalling the predominant presence of diatoms. Claustre et al. [51] interpreted an increase in values of both of these ratios as representative of increased proportions of diatoms. In addition to these two markers, the fatty acid, 16:4 ω 1, can be also used to assess the importance of diatoms. This acid is commonly found in diatoms [53, 54] but is very rarely encountered in other phytoplankton, and it can be used as a general marker for diatoms. In Trinity Bay, a detailed examination of the fatty acid composition of plankton samples collected during spring clearly showed the development of the spring diatom bloom with a maximum reached in all three markers in May (Fig. 3a). In addition to elevated amounts of 16 carbon fatty acids, diatoms produce large proportions of 20:5 ω 3. Dinoflagellates, on the other hand, generally contain higher proportions of 22:6 ω 3. The combination of those two fatty acids in a ratio, 22:6 ω 3/20:5 ω 3, produces a marker which reflects the predominance of dinoflagellates versus diatoms. It should be noted, however, that this marker only applies in environments such as Trinity Bay where dinoflagellates and diatoms are the major producers of 22:6 ω 3 and 20:5 ω 3, respectively

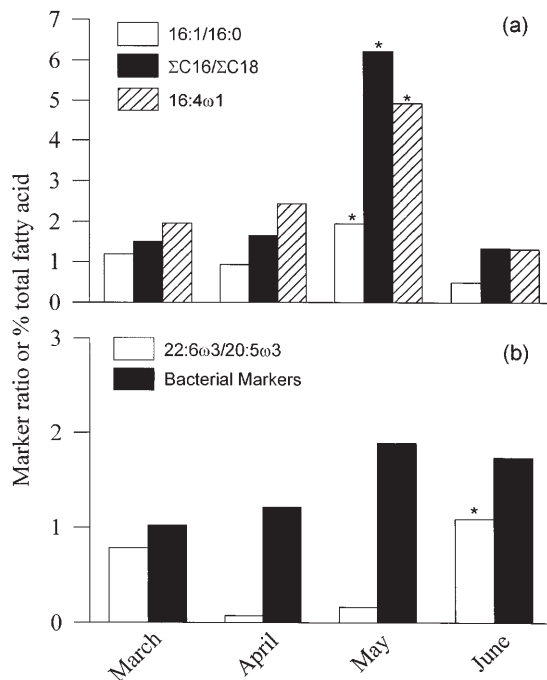


Fig. 3 a, b. Levels of biomarkers in plankton samples collected in Trinity Bay during the spring bloom of 1996 (After [50]). **a** Diatom markers and **b** dinoflagellate and bacterial markers. *Significantly different ($p < 0.05$) from the same marker value in all other months

[50]. In other environments, other microalgae may contribute significantly to the 22:6 ω 3 pool. In Trinity Bay, dinoflagellates were found to be a relatively more important source of fatty acid material before and after the spring bloom (Fig. 3b).

Several fatty acids, specifically 15:0, 17:0 and all branched fatty acids, are produced primarily by both aerobic and anaerobic bacteria [55–57] and the sum of those fatty acids has been used to estimate bacterial contributions [58–61]. A comparison of bacterial markers in plankton, sediment trap and sediment samples showed the lowest values, with little variation, in plankton samples (Fig. 3b), and the greatest bacterial levels in sediments. The sediment traps, containing partially degraded material, had bacterial marker levels intermediate between the other two sample types, and levels of bacterial markers increased with increasing period of deployment. However, there are conflicting theories concerning the usefulness of these markers and, for that reason, bacterial markers should only be employed with caution. For instance, in a recent paper, Harvey and Macko [57] did not find a correlation between total fatty acids attributed to bacteria and bacterial carbon, and they suggest that bacterial fatty acids only be used as qualitative tools to estimate bacterial contributions. Wakeham [62] also points out that fatty acids of common oceanic bacteria may not be compositionally different from planktonic fatty acids so that bacterial

contributions may not be easily discernible. On the other hand, Canuel and Martens [63] propose that bacterial biomass in coastal sediments can be calculated from bacterial markers such as those used here. In light of these conflicting theories, it seems that bacterial fatty acids should only be used to determine bacterial levels relative to other samples in the same study.

In marine environments, there is always interest in determining terrestrial plant contributions. Long-chain (> 24 carbons) fatty acids are often used as terrestrial plant indicators [61, 64, 65] but their analysis is problematic as those fatty acids do not elute within the maximum temperature limits of most polar columns. Alternatively, the fatty acids, 18:2 ω 6 and 18:3 ω 3, found in elevated amounts in most terrestrial plants [49], may be used as terrestrial markers [50, 66]. By examining terrestrial plant, pollen, riverine and plankton samples, an arbitrary threshold of 2.5% has been assigned to this indicator [50]. In this way, samples with values above this may be considered to have terrestrial material as a significant source of organic matter. Preliminary studies with compound specific isotope analyses offered support for this marker as the analyses showed that 18:2 ω 6 and 18:3 ω 3 in riverine samples were the most depleted of all fatty acids with $\delta^{13}\text{C}$ values (see Sect. 7 below) of -33.4 and -33.6‰ , respectively. In Trinity Bay, in spring and early summer, values of those markers were quite low in the plankton and sediment trap samples, but in late summer and fall, terrestrial markers reached a maximum in trap samples. Those markers also comprised almost 10% of total fatty acids in the sediments, suggesting that terrestrial material was preserved to a greater extent than marine fatty acids in sediments.

Zooplankton grazing is an important link between lower and higher trophic levels and fatty acid biomarkers may also be employed to determine the importance of zooplankton sources. Generally, herbivorous and omnivorous zooplankton feeding predominantly on phytoplankton contain elevated amounts of long-chain monounsaturated fatty acids within the wax ester lipid fraction [67–69]. Because of this, the sum of 20:1 and 22:1 fatty acids may be used as a zooplankton marker. In our study, zooplankton markers were highest in plankton samples, presumably because of the ample supply of phytoplankton as a food source during the spring bloom. These markers, however, also comprised approximately 6% of total fatty acids in both sediment traps and sediments, suggesting zooplankton-sourced material made a substantial contribution to the fatty acid pool. Over time, the zooplankton markers varied much like the terrestrial markers, with zooplankton becoming a more important source of sediment trap material in late summer and fall.

Information provided by examining these markers may be used to draw conclusions about carbon cycling and transfer of material through the food web. Plankton samples, containing fresh material, provide information concerning fatty acid sources over a very short time frame, allowing the development of the spring bloom to be monitored. Sediment traps provide a more integrated approach, collecting material over a period of several days to months. In the Trinity Bay study, fluxes of fatty acids through the water column were higher during the spring bloom and the traps predominantly captured diatom material at that time. In late summer and fall, terrestrial and zooplankton markers be-

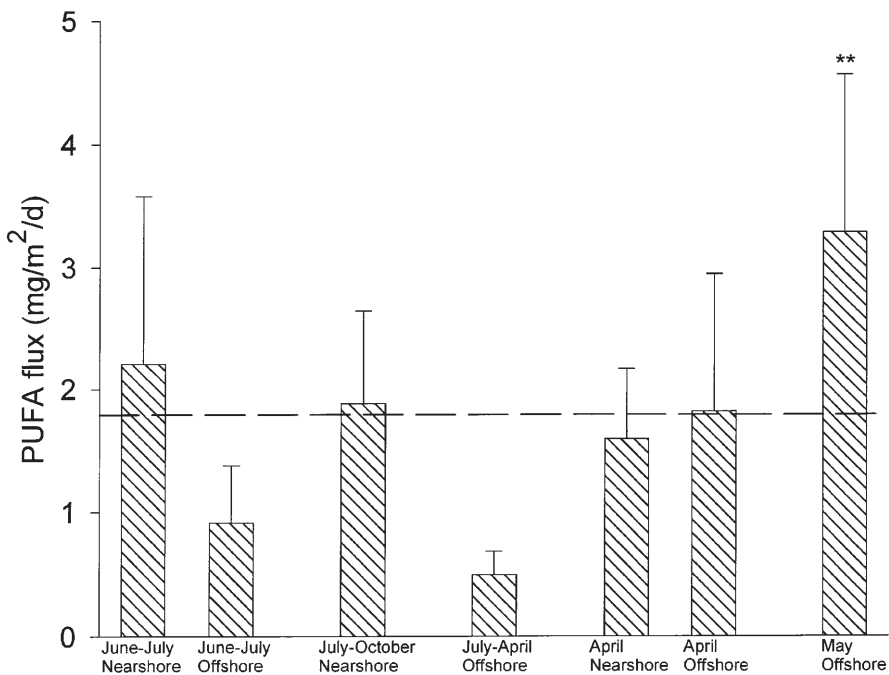


Fig. 4. Fluxes of particulate matter through the water column in Trinity Bay. Rates are the averages of traps at 3 depths (50, 75, 100 m) for polyunsaturated fatty acid inputs: $n = 9 - 11$. Error bars indicate one standard deviation above the mean for each flux; the dashed line is the annual mean flux. ** $p < 0.05$: Significantly higher than the annual mean flux

came important in the traps. Proportions of PUFA in both plankton and trap samples were high, indicating their efficient transfer to the sea floor. They are essential nutrients for animal survival and growth because they are necessary for normal membrane structure and function, especially at low temperatures [70]. Newfoundland is located in a subpolar oceanographic climate zone, and high levels of PUFA have been found in membrane and storage lipids in benthic organisms living in these cold waters [71].

In this area, there was a high flux of PUFA to the sea floor (Fig. 4) but there was very little preservation in sediments, indicating that the lower food web in this environment had an ample supply of lipids of high nutritional value and that these nutrients are very efficiently recycled. The efficient functioning of the lower trophic levels appears to have been the case for at least the past century as well, as marine lipids and fatty acids remained at about the same low level throughout a 30-cm core from the centre of Trinity Bay. This suggests that the decline in groundfish stocks in this area over the past three decades cannot be related to major shifts in the supply of energy or essential nutrients to the food web. However, the sink for all the marine lipid material sedimenting out of the water column remains to be established, since it is not the sediments and there has been a serious decline in groundfish in the area.

4 Sterols

Sterols are also potentially excellent biomarker compounds due to their stability and the diversity of their structures. They are present in all eukaryotes, and in marine material such as sediments, detection of 25 sterols or more is common. They share with phospholipids a structural function in membranes where, because of their unique hydrophobic and steric properties, they act as specific internal regulators of membrane fluidity and influence various membrane functions and membrane associated enzymes [72].

To determine sterols, a portion of the total lipid extract is saponified using methanolic potassium hydroxide, and sterols subsequently recovered in 2:1 hexane/chloroform. The sterols are converted to the corresponding trimethylsilyl (TMS) ethers using bis-*N,O*-(trimethylsilyl)trifluoroacetamide, BSTFA, and analyzed by capillary GC and GC with mass spectrometry. Reviews of relative retention times and mass spectra for sterol TMS ethers have been published [e.g. 73]. In some cases, sterol acetates, rather than TMS ethers, are the derivatives prepared for GC. Silica column chromatography of the total lipid extract may also be used instead of saponification to isolate the sterol fraction [74], or even sterol subclasses such as 4,4-dimethyl, 4-monomethyl and 4-desmethyl sterols [75], prior to derivatization. However, this approach only includes free sterols in the analysis, whereas by saponifying the total extract, sterols present as steryl esters are also detected.

In algae and in many invertebrates that feed directly on algae there are a wide variety of sterols that can be used for chemotaxonomic purposes and for food web tracing [76–79]. Their comparative resistance to degradation makes them even more valuable as long-term biomarkers than the lipid classes and fatty acids discussed above. Also they provide less ambiguous markers of terrestrial plants, phytoplankton, macroalgae, and of human sewage [e.g. 74]. For example, fresh domestic wastewater can be identified by high coprostanol/cholesterol and 24-ethylcoprostanol/ β -sitosterol ratios [80]. 24-Methylenecholesterol can be used as a marker for diatoms [3], and 24-ethylcholesterol, ethylcholest-5,22-dienol and 24-methylcholesterol for terrestrial plants [81], although the use of C_{29} sterols as higher plant markers requires caution, since certain algae also synthesize them [75]. Lastly, the ratio of corresponding saturated to Δ^5 -unsaturated species (stanol/stenol ratio) can be used to indicate the preservational state of marine material. The use of supporting evidence from other biomarkers or from floristic analyses greatly increases the certainty of sterol source assignment.

In our Trinity Bay study we found that the input of anthropogenic hydrocarbons was generally low and that the same was true of human sewage which could be ascertained from the sterol analyses. No coprostanol was detected at offshore sites. Very low levels of coprostanol may be present in sediments near towns, but its ratio to cholesterol and epicoprostanol make a sewage source unlikely. By contrast, coprostanol is the principal sterol (20 $\mu\text{g/g}$ dry weight sediment) in harbour sediments in St. John's, the major city on the island, where untreated sewage is also discharged. These results indicate that sewage input in the Trinity Bay area is either negligible, or is being efficiently degraded or dispersed.

Sterol biomarkers can be used to apportion inputs to ecosystems (Fig. 5), with specific sterols being assigned to different source organisms or categories (see also [82]). In sediments from our Trinity Bay study, total sterol concentrations in sediments averaged 24–44 $\mu\text{g/g}$ dry weight, with no discernible trend with depth, suggesting that sterols are well preserved. Source assignment for cholesterol and cholestanol, widespread in marine organisms, was done as follows. 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 source categories. 5β -Stanols are grouped separately; while not all 5β -stanols are unambiguous sewage indicators, they are all formed by bacterial biohydrogenation.

The C_{27} sterols (desmosterol, cholesterol) and C_{28} sterols (24-methylenecholesterol, 24-methylcholesta-5,22-dienol) predominated in the sterol composi-

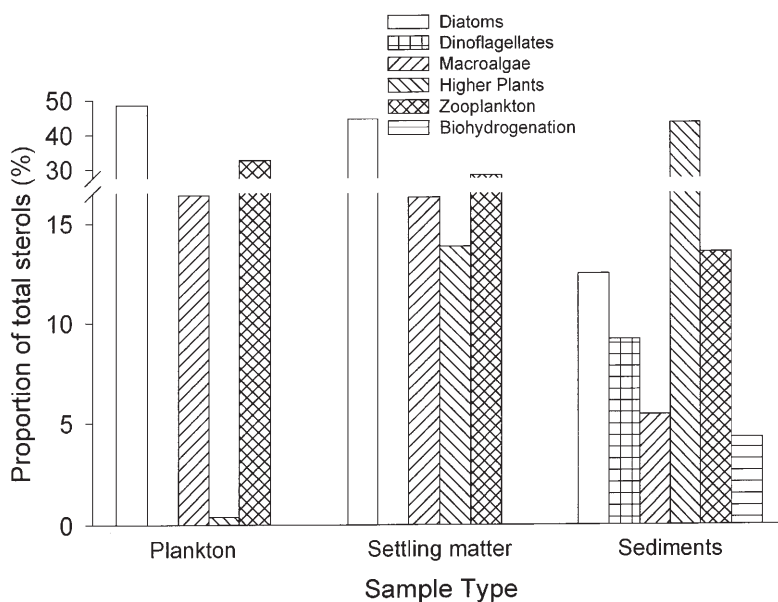


Fig. 5. Sources of organic matter in Trinity Bay as indicated by sterol biomarker composition. $n=8-10$. *Phytoplankton* (diatoms): cholesterol, cholestanol, desmosterol, brassicasterol, brassicasterol, 24-methylenecholesterol, 24-methylenecholestanol, dimethyldehydrocholestanol. *Dinoflagellates*: dinosterol, dehydrodinosterol, 4-methylcholesterol, dimethyldehydrocholesterol, cholesterol, cholestanol. *Macroalgae*: fucosterol, fucostanol, isofucosterol, isofucostanol, cholesterol, cholestanol. *Higher plants*: 24-ethylcholesterol, 24-ethylcholestanol, 24-methylcholesterol, 4-methylcholestanol, ethylcholesta-5,22-dienol, ethylcholesta-22-enol, 24-ethylcholestanol, $\text{C}_{30}\Delta^5$ steratrienol. *Zooplankton*: *trans*-22-dehydrocholesterol, *trans*-22-dehydrocholestanol, 24-nordehydrocholesterol, 24-nordehydrocholestanol, cholesterol, cholestanol, ocellasterol. *Biohydrogenation*: Coprostanol, epicoprostanol, ethylcoprostanol. Sterols due to biohydrogenation include both sewage markers and those formed by bacterial degradation

tion of net-tow and sediment trap samples, confirming their mainly marine origin [75]. The diatom sterols 24-methylcholesta-5,22-dien-3 β -ol, 24-methylenecholesterol and desmosterol are prevalent in spring/summer sediment trap material, which contained smaller proportions of terrestrial sterols. However, in sediments, C₂₉ sterols (e.g. 24-ethylcholesterol) associated with higher plants prevailed (Fig. 5). This indicates a much greater terrestrial contribution, even offshore, and indicates the potential for onshore activities to impact the marine environment. The dinoflagellate contribution (C₃₀ sterols) appears to have been well preserved in the sediments. No such contribution was detected in net-tow and sediment trap samples (Fig. 5), probably because those analysed for sterols were taken mainly in spring, while dinoflagellate numbers and biomass proportions increased in summer [50, 83].

5 Hydrocarbons

The last class of lipids being considered here are the hydrocarbons. Hydrocarbon markers include alkanes derived from algae or plant leaves, and polycyclic aromatic hydrocarbons (PAH) derived mainly from crude petroleum and fuel spills. PAH can also be found in combustion products of fuels such as heating oil, gasoline and wood. Coastal sediments act as the ultimate reservoirs for these compounds when they are transported unaltered through the water column.

While hydrocarbons represent only a small fraction of the organic matter present in marine sediments, they have proven to be a class of markers easily extracted by organic solvent (e.g. Soxhlet extraction with dichloromethane) and analysed (GC-MS or GC-FID). Hydrocarbons act as suitable markers for distinguishing different source inputs in marine sediments [84, 85] and for investigating the cycling of organic matter in the marine environment [86–89]. By adopting the approach of studying different classes of hydrocarbons simultaneously (*n*-alkanes, branched alkanes, aromatics etc.) stronger conclusions about carbon sources can be drawn [81, 90].

Certain hydrocarbons are recognized as hazardous environmental compounds. PAHs have been classified as “priority pollutants” because of their carcinogenic and mutagenic characteristics [91]. The health risk associated with PAH, together with the information they offer as environmental markers, justifies inclusion of these compounds in a general study of naturally occurring hydrocarbons (e.g. *n*-alkanes).

In our Trinity Bay study, cores taken from Hickman’s Harbour and near Clarenville indicate significant terrestrial inputs which have changed over time [92]. In Hickman’s Harbour there has been an increase in the $\Sigma nC_{26}-nC_{35}/\Sigma nC_{16}-nC_{25}$ ratio (nC_{26} represents a straight chain saturated hydrocarbon with 26 carbons; $\Sigma nC_{26}-nC_{35}$, sum of abundance of *n*-C₂₆ to *n*-C₃₅ hydrocarbons) during the past century (Fig. 6a) indicating greater leaf wax inputs [87] which may relate to wood cutting. ²¹⁰Pb dating of a core near Clarenville indicated a much faster sedimentation rate. Here, the $\Sigma nC_{26}-nC_{35}/\Sigma nC_{16}-nC_{25}$ ratio (Fig. 7a) indicates increased deposition of organic compounds from leaves since the 1950s. However,

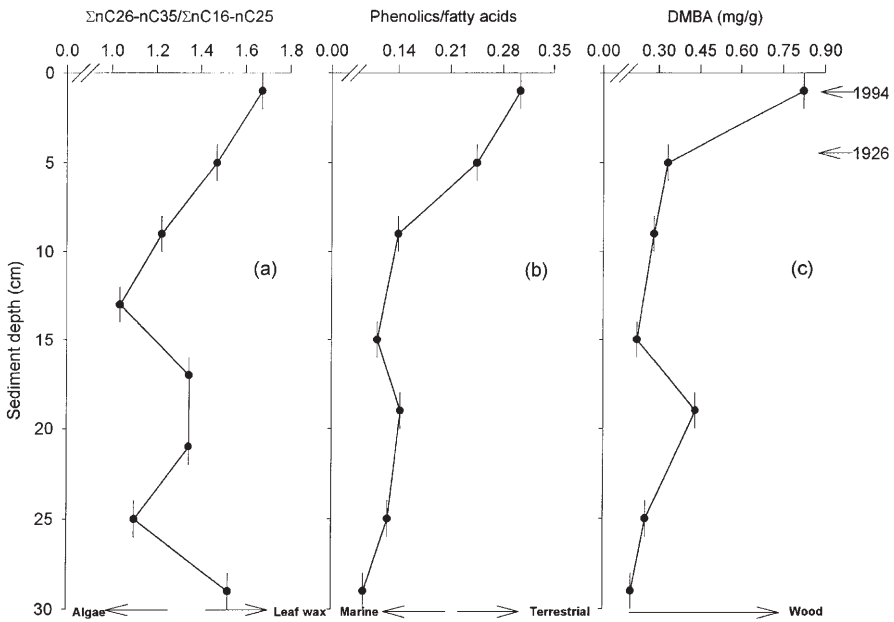


Fig. 6 a–c. Biomarkers in a Hickman's Harbour core. **a** High molecular weight/low molecular weight *n*-alkanes, **b** phenolic/saturated+branched fatty acid ratio determined by TMAH thermochemolysis, **c** 3,4-dimethoxybenzoic acid methyl ester (DMBA). ^{210}Pb dates were obtained for the top 6 cm of the core. (After [92, 106])

since the mid-1980s there may have been a decline in this source. The total hydrocarbon profile and the high molecular weight/low molecular weight *n*-alkane profiles in the two cores were very similar suggesting the major hydrocarbon source in the area is related to terrestrial plants.

Given the amount of oil field development around Newfoundland, it is critical to establish baseline PAH levels. The input of PAH in this area is apparently low currently except in the vicinity of Hickman's Harbour, where it may be significant. Low levels of various PAH pollutants were identified and their concentrations summed [92]. Total PAH in the Trinity Bay area averaged 0.1 $\mu\text{g/g}$ which is very much lower than St. John's Harbour where a value of 17 $\mu\text{g/g}$ was obtained [93]. St. John's is the biggest city in the Province. The concentration of PAH in Hickman's Harbour surface sediments (0.6 $\mu\text{g/g}$) was notably higher than in the other Trinity Bay cores suggesting some channeling from the watershed. Among the compounds we examined, the major organic pollutants were the products of wood burning which can be transported mainly through the atmosphere and then deposited by precipitation. This is expected given the amount of wood burning for domestic fuel in the area. The major pyrolytic influences were indicated by the dominance of parental PAH over alkylated PAH [94], and the prevalence in the samples of fluoranthene:pyrene ratios greater than 1 [92]. These inferences have been clearly documented in other studies [81, 89]. Finally, the close resemblance of PAH profiles, together with the similarity

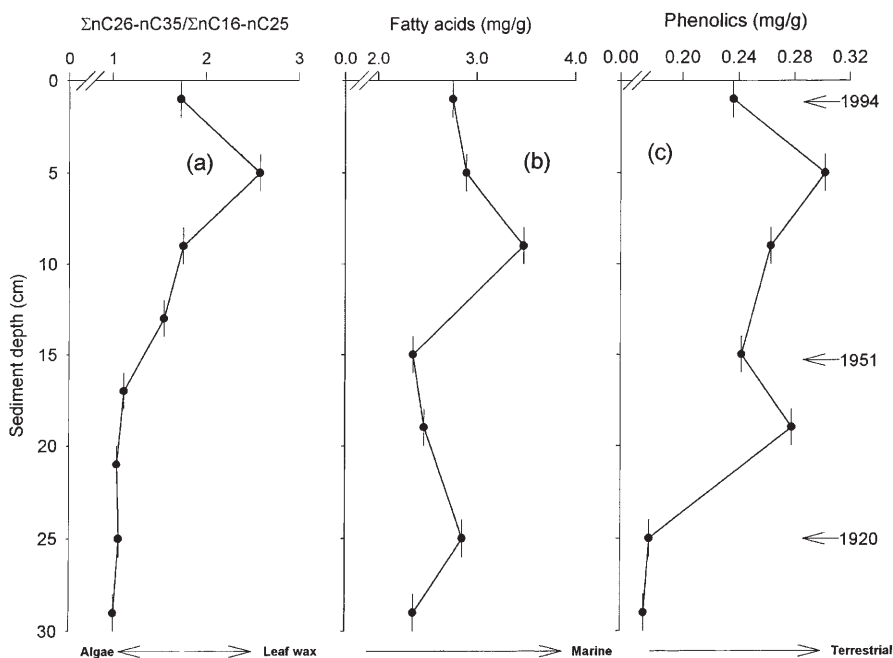


Fig. 7a-c. Profiles of organic classes in a core taken near Clarendville. **a** High molecular weight/low molecular weight *n*-alkanes, **b** total saturated+branched fatty acids, and **c** total phenolics determined by TMAH thermochemolysis. ^{210}Pb dates were obtained for the top 26 cm of the core. (After [92, 106])

in the phenanthrene/alkylated phenanthrene ratio in all samples, indicate PAH have the same source for the whole area.

6 Pyrolysis Products

Pyrolysis products used as markers include the phenolics. Analytical pyrolysis is a small scale, whole sample, analytical technique which involves the thermal fragmentation of complex organic macromolecules into a range of smaller molecules (pyrolysis products) by rapid application of heat in the absence of oxygen. The products are then identified and quantified by GC-MS to determine the composition of the original sample. The technique can be particularly useful for characterization of complex, non-volatile bio- and geopolymers present in soils, forest humus and aquatic sediments. It is known that simple pyrolysis releases many compounds having phenolic and carboxylic groups which are difficult to analyze by GC. A new technique recently reported for flash pyrolysis with in situ derivatization using tetramethylammonium hydroxide (TMAH) [95] has been shown to be a thermally assisted chemolysis rather than pyrolysis (also called TMAH thermochemolysis) [96, 97]. TMAH not only methylates polar pyrolysis products but also assists in bond cleavage [96]. The

technique is now widely used in studying lignin in wood [98, 99] and humic substances in soils [100, 101] and has now been extended to marine sediments [102]. We have adopted the off-line sealed tube technique of McKinney et al. [99] which uses a lower chemolysis temperature (300°C for 30 min). After excess TMAH has been removed, the products can be extracted with methylene chloride and analyzed by GC-MS [102].

One side reaction which occurs with TMAH/ heat is the formation of benzenecarboxylic acid methyl esters from lignin [103] and from aromatic aldehydes [104, 105]. Therefore our study summed all phenolic-containing products and their methylated derivatives (or "phenolics") observed in marine sediments (Figs. 6–8). Phenolic compounds are abundant in herbs, shrubs and trees. Phenolics can be released during degradation of lignin, from decomposition of leaves and are common plant metabolites.

Lignin is the connective tissue found in plants that gives them rigidity: it comprises 25–30% of the wood of trees and is a generic name for complex irregular phenolic polymers. Lignin is classified into three main groups on the basis of their structural monomer units. One unit common in both hardwood and softwood is coniferyl alcohol. TMAH thermochemolysis products of this unit include 3,4-dimethoxybenzoic acid methyl ester (DMBA), and 3,4-dimethoxybenzaldehyde [106]. DMBA's precursor, vanillic acid, has been used as a lignin-derived marker in marine sediments on the outer Great Barrier Reef

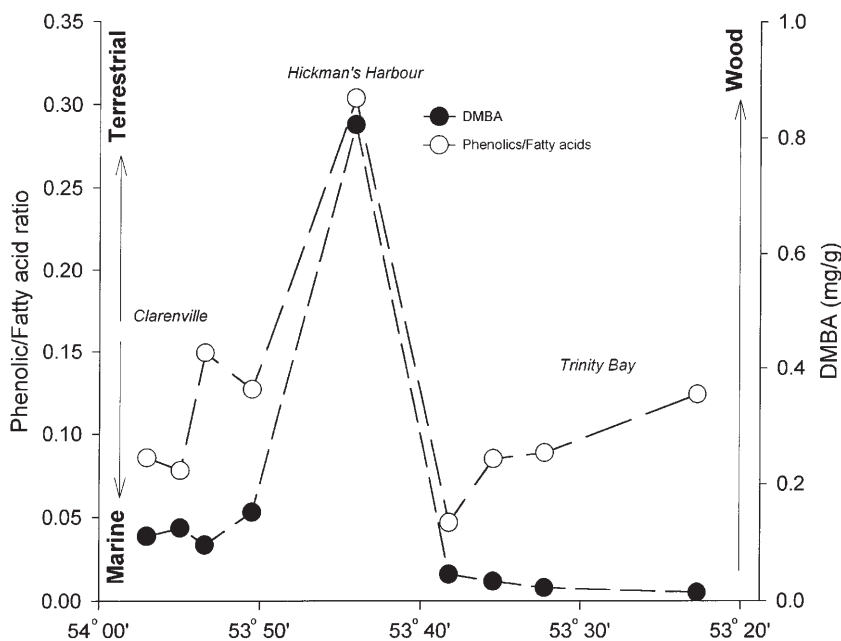


Fig. 8. Ratio of total saturated + branched fatty acids and total phenolics and 3,4-dimethoxybenzoic acid methyl ester content of surface sediments determined by TMAH thermochemolysis. (After [106])

[107]. Pulchan [106] thus used DMBA as a derived marker of vascular plant material (trees and scrubs) in offshore sediments in Trinity Bay (Figs. 6–8). Such terrestrial organic matter may play a significant role in the productivity of marine ecosystems by adding to the marine carbon pool.

Fatty acid methyl esters are another abundant group of products produced by TMAH thermochemolysis. Under these conditions fatty acids of triacylglycerols and other lipids are effectively esterified [97]. But only the saturated/branched fatty acids can be used as reliable biomarkers with this analysis. Unsaturated fatty acids tend to isomerize and degrade under the strong basic conditions using TMAH and heat [108].

In our Trinity Bay study, the TMAH thermochemolysis products as well as the alkane distribution indicated significant terrestrial inputs. The thermochemolysis products included phenolic and saturated/branched fatty acids which were further characterized for their stable carbon isotope composition [102, 106]. The results suggested that the fatty acids were of marine origin while the phenols were derived from terrestrial plants. Although total fatty acid and total phenolic concentrations both increased near Hickman's Harbour, the ratio of their concentrations clearly shows the influence of terrestrial plant inputs there (Fig. 8). The 3,4-dimethoxybenzoic acid methyl ester (DMBA) data further define the source as being derived from terrestrial plants (i.e. the wood and leaves of trees or scrubs). There is five times the concentration of this marker near shore than in Trinity Bay demonstrating the greater influence of wood inputs near the land-margin. However, the widespread occurrence of significant levels of terrestrial markers in the marine sediments indicates that land-derived contaminants could easily become widely dispersed in this system.

Thermochemolysis data from cores from Hickman's Harbour and adjacent to Clarendville [106] also indicate that the degree and type of terrestrial input have changed noticeably over the past century. High concentrations of total phenolics, total fatty acids and total hydrocarbons were found near the surface at Hickman's Harbour and to a smaller extent near the middle of the core. The increase in total fatty acids found near the surface at Hickman's Harbour is smaller than the increase in total phenolics so that the phenolic/fatty acid ratio also increases near the surface (Fig. 6b). The lignin marker (DMBA) and the *n*-alkane ratio point to the significant influence of terrestrial plants, specifically leaf and wood inputs. The higher concentration of marine-derived saturated fatty acids is probably a result of higher preservation in these organic rich sediments. The higher wood contribution to the shallower sediments around Random Island in comparison with the last couple of centuries, suggests a recent change in the nature of terrestrial inputs. This could be a consequence of the natural evolution of the surrounding terrestrial ecosystem, or of human activity such as logging and milling. In 1911 there were 14 sawmills in Hickman's Harbour. The increase in all the organic classes and the biomarkers near the middle of the core is interesting as it likely predates the discovery of Hickman's Harbour by Europeans. However, Hickman's Harbour was probably an area of Indian encampment, and in fact, was one of the last homes of the Beothuk Indians on the east coast.

Due to faster sedimentation rates near Clarenville we have many more samples dating from the last century, and the marked increase in organic compounds derived from terrestrial plants in the 1920s is even clearer as indicated by an increase in phenolics at 20 cm core depth (Fig. 7c). The marked increase in the $\Sigma nC_{26}-nC_{35}/\Sigma nC_{16}-nC_{25}$ ratio (Fig. 7a) since the 1950s suggests greater wood cutting for fuel since then. The fact that the profile of the marine-derived fatty acids is completely different to that of the phenolics or of the hydrocarbons, suggests that levels of land use changes have, at most only weakly impacted the pattern of marine biogenic productivity in Trinity Bay.

7

Carbon Isotope Chemistry of Biogenic Compounds

The isotopic composition of organic compounds has been used to speculate on sources of sedimentary organic matter, and on a wide range of paleo-environmental conditions, including temperature, water column stratification, and dissolved inorganic carbon (DIC) concentrations [e.g. 109–112]. The organic matter that is eventually stored in sediments, however, was originally synthesized by various organisms at different trophic levels using a number of distinct biochemical pathways. These differences, as well as factors such as temperature, growth rate, and DIC concentrations contribute to the considerable variation in isotopic composition observed in organic matter. Our limited understanding of these processes, as well as of the distribution, cycling and degradation of organic compounds, means that studies of the isotopic composition of modern environments is important for correct interpretation of fossil isotopic signatures.

Our studies of cold coastal ecosystems of Newfoundland have combined molecular biomarker characterization with the determination of carbon isotope ratios of individual compounds during spring blooms. These studies were conducted to elucidate the transfer of primary photosynthate in the water column to benthic environments. Such studies were also undertaken to measure the range of fatty acid isotopic compositions in a modern depositional environment, and to test the use of isotopic compositions as tracers of fatty acids in settling particles. Compound-specific carbon isotope determinations were made on fatty acids as well as biogenic hydrocarbons (alkanes, highly branched isoprenoids: [113]). Compound-specific carbon isotope measurement can be performed on fatty acids esterified using BF_3 -methanol. Compound-specific GC-combustion-isotope ratio mass spectrometry (GC/C/IRMS) analyses of fatty acid methyl esters can be undertaken using a VG Isochrom system equipped with a gas chromatograph. Standardization is accomplished by comparing integrated $^{13}C/^{12}C$ for each compound peak with similar ratios from pulses of reference CO_2 gas introduced before and after the sample chromatographic window. The accuracy of this procedure is tested by co-injection of fatty acid carbon isotopic standards. Measured carbon isotope compositions for esters can be corrected according to the procedure outlined by Abrajano et al. [114], and reported as $\delta^{13}C$:

$$\delta^{13}C_{\text{sample}} = 1000 * \{({}^{13}C/{}^{12}C_{\text{sample}} / {}^{13}C/{}^{12}C_{\text{PDB}}) - 1\}$$

$\delta^{13}\text{C}$ values, in parts per thousand, are used to describe the small variations in the relative isotope abundances. The key step in the isotopic segregation of carbon is at the point of fixation into plants. For both terrestrial and aquatic plants, photosynthetic fractionation of carbon isotopes arises from differential diffusion of isotopic species of CO_2 , H_2CO_3 or HCO_3^- , and from catalytic reactions that vary depending on the fixation pathway. Most marine phytoplankton utilize the C_3 fixation pathway (Calvin-Benson cycle), resulting in bulk $\delta^{13}\text{C}$ values in the range -20 to -30‰ . The latter value includes the compounding effect of diffusive fractionation and various environmental parameters such as DIC concentrations. The carbon isotopic composition of individual compounds in photosynthesizers also depends on fractionation steps at major branching points of compound synthesis. For example, it has long been known that lipids are generally depleted in ^{13}C compared to bulk $\delta^{13}\text{C}$ values of organisms [e.g. 115]. Carbon isotope fractionation in fatty acids, in particular, could occur during the formation of acetyl-CoA and subsequent chain elongation.

Bulk carbon isotope values recorded across spring diatom increases in the cold coastal environments of Newfoundland were reported by Ostrom et al. [116] for Conception Bay (Fig. 1, inset) which is an environment very similar to nearby Trinity Bay. They noted carbon isotope shifts that are consistent with lowered DIC concentrations at the peak of the bloom. This depleted DIC concentration can lead to ^{13}C -enriched organic matter because lower DIC availability also leads to lower isotopic discrimination (i.e. lower isotopic selectivity). Our work focused on compound-specific measurements on individual hydrocarbons and fatty acids in Conception Bay. Bieger et al. [113] noted carbon isotope variations for alkanes and highly branched isoprenoids (HBI) that are consistent with the variations noted by Ostrom et al. [116]. Hydrocarbons generated at the peak of the bloom tend to be enriched in ^{13}C , again suggesting lower isotopic discrimination when DIC substrate concentration is low.

The work of Bieger et al. [113] provided the additional insight that different compounds may record generation at different stages of the bloom. Although this is expected on physiological and biochemical grounds, the work of Bieger et al. [113] was the first to document this isotopically. Most algal products found in plankton and sediments, such as heneicosahexaene (HEH) and pristane, had isotopic compositions between -25 and -28‰ . The HBI alkenes, however, were consistently depleted by at least 2‰ (mean $\delta^{13}\text{C} = -33\text{‰}$) relative to most other marine biogenic compounds. Among the four pairs of HBI alkene isomers, the later eluting isomer was, in each pair, consistently enriched in ^{13}C . The C_{20-25} alkenes found in the near-shore sediments were all significantly enriched in ^{13}C (mean $\delta^{13}\text{C} = -20.3\text{‰}$) relative both to co-occurring hydrocarbons and to the C_{25} HBI alkenes in the mid-bay samples. The HBI alkenes in crab and scallop samples were isotopically similar to the same compounds in plankton tows and sediments. Squalene was relatively enriched (mean $\delta^{13}\text{C} = -24\text{‰}$), whereas the C_{25} HBI alkenes were all strongly depleted (from -30.6 to -40.5‰). The average $\delta^{13}\text{C}$ of the *n*-alkanes in spring plankton fell over the course of the bloom from -23.7‰ to -29.6‰ . The long-chain *n*-alkanes ($>\text{C}_{25}$) found in all sediment samples were consistently depleted in ^{13}C ($\delta^{13}\text{C} < -30\text{‰}$) compared with shorter chain-length homologues. Bieger et al. [113] speculated that the ^{13}C -de-

pleted highly branched isoprenoids were generated prior to the bloom, possibly largely utilizing respired carbon in deeper portions of Conception Bay. While compound-specific carbon isotope variations in alkanes and other biogenic compounds appear to track the effects of decreasing carbon substrate across the spring bloom (i.e. ^{13}C enrichment at the peak of the bloom), there was also isotopic evidence that the short and long chain *n*-alkanes were derived from isotopically unrelated sources.

Our most recent work on fatty acids in Conception Bay [117] has provided additional details to the overall observations made for bulk carbon and individual hydrocarbons discussed above. We combined detailed molecular characterization with carbon isotopic measurements to describe both the temporal and depth variations of primary production during the spring bloom. The isotopic compositions of the fatty acids are summarized in Fig. 9. Bulk spring bloom particulate organic matter in Conception Bay has a $\delta^{13}\text{C}$ value between -24‰ and -26‰ [116]. Since lipids are normally expected to be depleted relative to

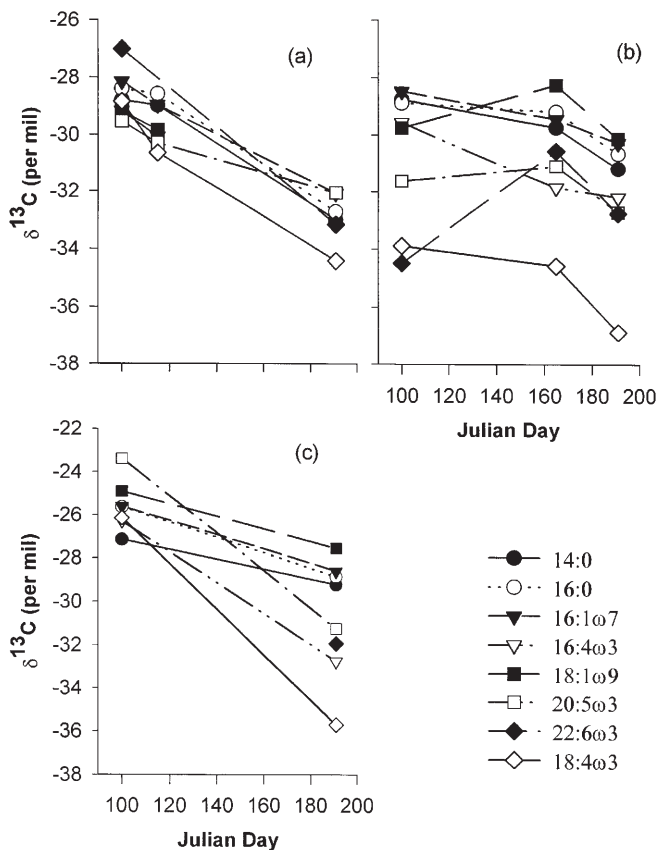


Fig. 9 a–c. Carbon isotope composition of individual fatty acids measured across the 1996 spring bloom in Conception Bay, Newfoundland. **a** Horizontal net-tow **b** 80 m depth, HgCl_2 -poisoned sediment trap and **c** 220 m depth, HgCl_2 -poisoned sediment trap. (After [117])

total biomass by roughly 5‰ as a result of fractionation during early synthetic stages [115], the isotopic compositions of fatty acids in this study are generally consistent with the expected range for local phytoplankton blooms. A notable exception to this observation is the $\delta^{13}\text{C}$ values recorded for 18:4 ω 3 especially at the waning stages of the bloom. This observation was first noted in Trinity Bay mussels by Abrajano et al. [114], who suggested substantial fractionation during desaturation or a possible additional source for 18:4 ω 3. Note that the compound-specific $\delta^{13}\text{C}$ values for fatty acids overlaps with those previously reported by Abrajano et al. [114] for Trinity Bay mussels. In general, unsaturated fatty acids are depleted in ^{13}C compared to saturated counterparts, but this is most apparent in samples collected at the waning stages of the bloom. Here, the ω 3 fatty acids are clearly systematically depleted in ^{13}C compared to the saturated counterparts, and this was most evident for 18:4 ω 3. Other $\delta^{13}\text{C}$ observations made on Trinity Bay mussels [114] also showed consistent ^{13}C depletion in PUFA, possibly resulting from *de novo* carbon isotope fractionation or additional sources for PUFA (specifically the ω 3 fatty acids). Although previous authors have found fatty acid desaturation to be associated with a preferential loss of ^{13}C [118], the lack of a similar isotopic depletion among these highly unsaturated fatty acids suggests that relatively little isotopic discrimination occurs during desaturation.

The durability of the isotopic signatures of fatty acids in the water column of Conception Bay is demonstrated by the consistency of the isotopic compositions in plankton, particulate and benthic macrobiota. It is evident that the absolute $\delta^{13}\text{C}$ values and their total range overlap within the analytical variability. More importantly, the intermolecular variations (e.g. depleted ^{13}C in ω 3 fatty acids) noted in plankton are largely preserved in the trap materials down to 220 m depth (Fig. 9c). These observations imply that $\delta^{13}\text{C}$ individual compound “signatures” of primary producers can be traced into the benthic environment, although sedimentary signatures are a temporal average.

The most noteworthy temporal carbon isotopic trend observed in fatty acids across the spring bloom is the more depleted $\delta^{13}\text{C}$ values subsequent to the main phase of the bloom. This observation is consistent with those noted above for biogenic hydrocarbons (e.g., alkanes, HBI) by Bieger et al. [113]. The temporal trend in isotopic compositions was possibly due to either lowered DIC concentrations at the peak of the bloom or very high growth rates of phytoplankton for the same period [113]. As shown by Laws et al. [119], the isotopic composition of primary producers is a function of growth rate, in that the isotopic discrimination factor involved in the assimilation and fixing of DIC is reduced during periods of intense growth. Thus, compounds synthesized before or after a bloom, when growth rates are lower, would be expected to have lower $\delta^{13}\text{C}$ values. If the $\delta^{13}\text{C}$ values arose during fatty acid synthesis, the clearer $\delta^{13}\text{C}$ distinction between saturates and PUFAs observed at the end of the bloom would imply that the depletion in ^{13}C is likely related to growth rate. Changing substrate DIC concentration is more likely to shift $\delta^{13}\text{C}$ for individual fatty acids to the same extent.

Compound-specific isotope analyses were also used to help determine the origins of some of the important hydrocarbons and thermochemolysis pro-

ducts isolated from Trinity Bay sediment core sections [92, 102]. Phenols and fatty acids produced by thermochemolysis of sediments were characterized for their stable isotope composition and then compared with samples taken from the topsoil near the marine sites. In general, the results suggested that the fatty acids were of marine origin while the phenolics were derived from lignin.

8 Chemometrics

Chemometrics are the use of mathematical methods in chemistry, such as the use of applied statistics for chemical analyses. They are widely used as a tool in pure research and in industry [120] for the investigation of large amounts of data. Chemometrics are used in experimental design such as the factorial type of experiment where it is determined how the result is affected by simultaneous changes in different factors or variables. The major advantage of the factorial experiment compared with the single-variable-at-a-time method is that it is sensitive to interactions between factors [121]. This multivariate approach has been used to optimize autoinjection procedures and column and injector temperature programs in the gas chromatographic analysis of marine lipids [29].

The application of chemometrics to marine biomarker data is becoming increasingly common as it permits data reduction and an objective interpretation of the results. When there are only a few samples and a few measurements made on each sample then a correlation matrix can be quite useful. For example, Parrish [122] used a correlation matrix to demonstrate the decoupling of dissolved and particulate lipid classes during a spring bloom. This approach can be especially interesting when different types of chemical data are compared or when biological data are compared with chemical data. Derieux et al. [36] found a strong relationship between TAG and 20:5 ω 3 and between TAG and 22:6 ω 3 in marine dissolved and particulate matter which they attribute to preferential degradation of PUFA in energy reserves. By correlating fatty acid analyses with biovolumes derived from seston population analysis, Parrish et al. [44] were able to confirm 14:0 and the ratio 16:1/16:0 as markers for diatoms, 18:5 ω 3 and 20:4 ω 6 as microzooplankton markers, and 15:0 and 22:6 ω 3 as cryptophyte markers. A more sophisticated use of correlation coefficients involves the multivariate technique of cluster analysis. This type of analysis is available in software packages such as Minitab and its purpose is to classify observations into groups when the proper grouping is initially unknown. Colombo et al. [123] used cluster analysis on organic classes measured in settling particles in the Laurentian Trough. A dendrogram based on the correlation coefficients shows the data falling into a phytoplankton group and a zooplankton group and it suggests that lipids and amino acids are the principal contribution of zooplankton to the vertical flux of carbon.

In the marine biomarker field, principal components analysis (PCA) is gaining in popularity as a powerful data reduction procedure. This multivariate technique handles a large amount of variables at the same time, instead of the traditional pairwise correlation studies. With the use of autoinjectors on gas chromatographs and integration software that can be directly linked with

spreadsheets it is possible to quickly generate large amounts of data that would be very difficult to interpret by correlation analysis. In such a situation, a multitude of significant correlations can be generated and it is very difficult to visualize and then utilize the results. PCA, on the other hand, gives a simple graphical representation of the similarities in the data set which allows all the observations (variables) to be considered at the same time. It establishes the principal characteristics of a set of variables on a series of samples by systematically reducing all the original variables to a smaller more coherent set of derived variables (principal components) that capture most of the information contained in the original variables [124]. In the process, it maximizes the variance accounted for in the original variables. It is similar to other procedures such as factor analysis, discriminant analysis, canonical correlation analysis, principal coordinate analysis and correspondence analysis. PCA is more commonly used with lipid data; however, what was probably the first multivariate study of marine lipid data was performed using correspondence analysis [125].

PCA can be easily performed on a data set using statistical software packages such as Minitab and the results displayed using plotting packages such as SigmaPlot (Figs. 10–12). Normality of the data is not required for PCA, however, both Jeffries and Lambert [126] and Mayzaud et al. [127], early users of PCA of lipid data, transformed their percentages. While a whole series of principal components is calculated, often just the first two explain a large proportion (>70%) of the total variance. Thus, by plotting the coefficients of these two principal components a good approximation of the distribution of the observa-

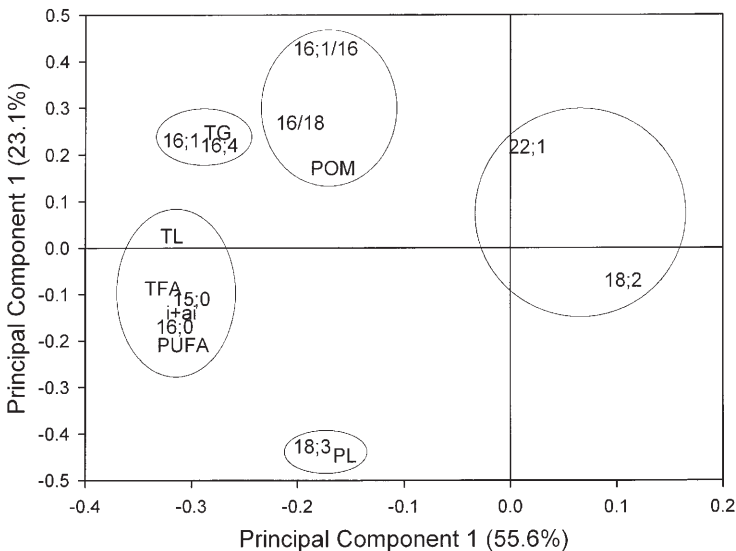


Fig. 10. Principal components analysis (PCA) of Trinity Bay lipid data. Fatty acid and lipid class concentrations in plankton tows from 3 stations were analysed. *PL*: phospholipid, *POM*: particulate organic matter, *PUFA*: Σ polyunsaturated fatty acids, *TG*: triacylglycerol, *TL*: total lipid, 16:1: 16:1 ω 7, 16:1/16: 16:1/16:0, 16:18: $\Sigma C_{16}/\Sigma C_{18}$, 16:4: 16:4 ω 1, 18:2: 18:2 ω 6, 18:3: 18:3 ω 3, 22:1: 22:1 ω 11

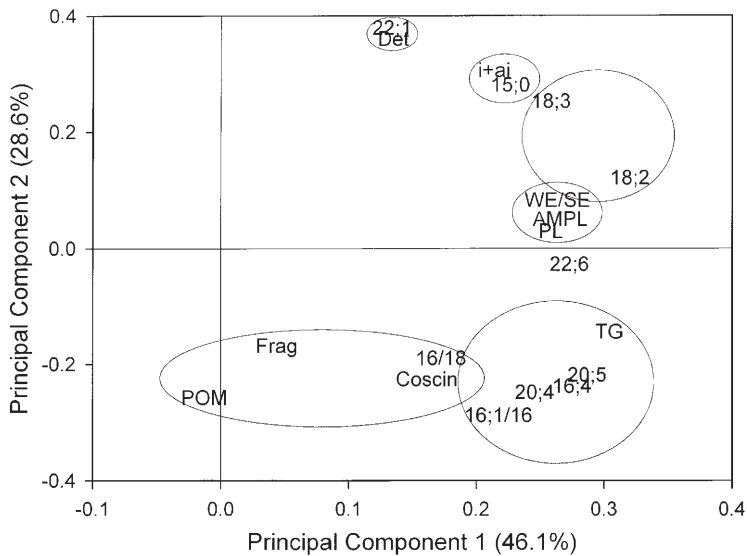


Fig. 11. Principal components analysis of lipid and microscopy data from the water column in Trinity Bay. All data used were concentrations per dry weight. AMPL: acetone-mobile polar lipids, *Coscin*: *Coscinodiscus*, *Det*: detritus, *Frag*: *Fragilaria*, *i+ai*: *iso+anteiso*, *PL*: phospholipid, *POM*: particulate organic matter, *TG*: triacylglycerol, *WE/SE*: wax and steryl esters, 16:1/16: 16:1/16:0, 16/18: $\Sigma C_{16}/\Sigma C_{18}$, 16:4: 16:4 ω 1, 18:2: 18:2 ω 6, 18:3: 18:3 ω 3, 20:4: 20:4 ω 6, 20:5: 20:5 ω 3, 22:1: 22:1 ω 11, 22:6: 22:6 ω 3

tions is obtained. The plot allows all variables to be considered simultaneously to determine groups and outliers. Groups can be encircled manually [e.g. 126, 127] or rays can be drawn to the variables as used by Colombo et al. [82] in a study of particulate lipids in the Laurentian Trough. There are clustering algorithms that can be used, but Dunteman [124] suggests that visual clustering is as good. We prefer to use a software drawing procedure that limits the group to the shape of a circle or an ellipse that can stretch only along the axes of the principal components (Figs. 10–12). If the first two principal components do not account for a substantial amount of the variation then it is useful to supplement with information from the third one. This could be done by simply adding the sign of the coefficient to each point on the original plot [124], by replotting the coefficients of the first principal component against those of the third principal component [127], or in a three-dimensional plot [125]. If sampling was undertaken in a continuous spatial or temporal sequence then the plotting of the scores of the principal components for each sample can be very useful in addition to plotting the coefficients. In this way Mayzaud et al. [127] used PCA on arcsin transformed data to establish seasonal succession in seston in a small marine inlet, in terms of both size and chemical characteristics. Colombo et al. [82] plotted both on the same graph and showed that a large proportion of the total variance in their lipid biomarker data was related to a terrestrial-marine or vascular plant-phytoplankton gradient in the Laurentian Trough.

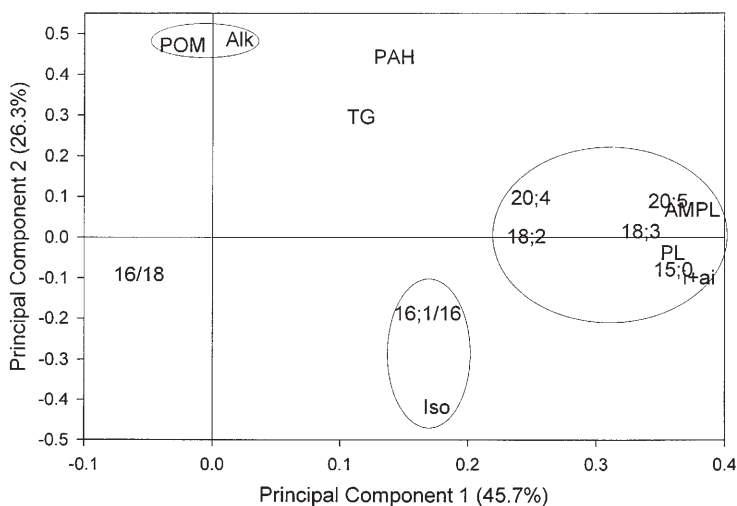


Fig. 12. Principal components analysis of sediment data from Trinity Bay. All data used were concentrations per dry weight. *Alk*: Σ alkanes, *AMPL*: acetone-mobile polar lipids, *i + ai*: *iso + anteiso*, *Iso*: Σ isoprenoid hydrocarbons, *PAH*: Σ polycyclic aromatic hydrocarbons, *PL*: phospholipid, *POM*: particulate organic matter, *TG*: triacylglycerol, 16;1/16: 16:1/16:0, 16/18: $\Sigma C_{16}/\Sigma C_{18}$, 18;2: 18:2 ω 6, 18;3: 18:3 ω 3, 20;4: 20:4 ω 6, 20;5: 20:5 ω 3

We found very little difference between using percentage data and arcsin transformed data, but it is important to note that there was a substantial difference in the appearance of the plots of the first two principal components depending on whether percentage or concentration data were used. Shown in Figs. 10–12 are PCA of concentration data. In an analysis of lipid class and fatty acid data from three stations in Trinity Bay (Fig. 10) the first two principal components account for a large amount of the variation in the original data (79%). The first axis separates microorganisms (16:1 ω 7 and PUFA from microalgae and *iso+anteiso* acids and 15:0 from bacteria) and higher organisms (22:1 ω 11 from zooplankton and 18:2 ω 6 from terrestrial plants). The second axis shows a weaker separation of plant lipids. Diatom markers generally show a more positive loading on the second axis with 16:1/16:0 showing the most positive loading and terrestrial markers generally show a more negative loading with 18:3 ω 3 showing the most negative loading. The grouping of the bacterial markers with PUFA and 16:1 ω 7 suggests that bacteria are associated with phytoplankton. The association of triacylglycerols with 16:1 ω 7 and 16:4 ω 1 suggests diatoms are the major source of triacylglycerols and that these acids are significantly associated with storage. The central position of POM shows the multiple contributions but its closeness to diatoms markers signifies their importance as a source in plankton.

PCA of fatty acid and lipid class data for net-tows and traps from various stations consistently showed total lipids and 16:0 grouping together reflecting the ubiquity of this acid in acyl lipids. 15:0 and *iso + anteiso* acids always grouped

together confirming that 15:0 has an important bacterial source. In addition, Σ PUFA were also always associated with the bacterial group suggesting bacterial activity was associated with algae. Also 16:4 ω 1 and TG were usually associated in tows and traps suggesting diatoms are a major source of TG throughout the water column. 16:1/16:0 and $\Sigma C_{16}/\Sigma C_{18}$ were consistently located near organic matter. Similar patterns were found in the traps whether the data were expressed as concentrations or fluxes. On the basis of these similarities and the fact that lipolysis indices were similar in the traps and the tows, the lipid class and fatty acid data from tows and traps were combined and then compared with biovolume data estimated by microscopy (Fig. 11). The appearance of this plot is quite similar to that obtained when PCA is performed on sediment trap data alone.

For the net-tow and sediment trap data combined (Fig. 11) the first 2 PCs account for 75% of the variation in the original data. PC1 separates pennate diatoms (*Fragilaria*) and terrestrial material (18:2 ω 6 and 18:3 ω 3) while PC2 separates centric diatoms (*Coscinodiscus*) from detritus and zooplankton material (22:1 ω 11). The location of the bacterial markers (15:0 and *iso + anteiso* acids) suggests they are associated both with terrestrial material and with detritus. Triacylglycerols are again located near the diatom markers. *Coscinodiscus* is also located close to the diatom markers ($\Sigma C_{16}/\Sigma C_{18}$, 16:1/16:0, 16:4 ω 1) and two PUFA which are quite prominent in the genus [128]. 22:6 ω 3 which is more prominent in dinoflagellates and flagellates than in diatoms, is separated from other fatty acids. The location of the centric and pennate diatoms near POM underlines the importance of diatoms as overall contributors to organic matter in the water column. Wax and steryl esters, acetone-mobile polar lipids and phospholipids are centrally located indicating the diversity of sources, although wax and steryl esters are located closer to 22:1 ω 11 and phospholipids to the long-chain PUFA, reflecting their major sources.

Hydrocarbon analyses performed on the sediments from Trinity Bay [92] were included in the analysis of the sediment data (Fig. 12). Here, the first 2 PCs account for 72% of the variation. PC1 separates diatoms ($\Sigma C_{16}/\Sigma C_{18}$) and bacteria (*iso + anteiso*) while PC2 separates marine (isoprenoid hydrocarbons) and terrestrial (*n*-alkanes) material. The isoprenoid hydrocarbons consisted mainly of C_{25} highly branched alkenes while the *n*-alkanes were dominated by *n*- C_{27} and *n*- C_{29} [92]. The latter were located with POM signifying the importance of terrestrial plant material in sediments. The diatom marker 16:1/16:0 groups with the isoprenoids indicating these contributions are linked. 15:0 and *iso + anteiso* come together suggesting bacteria are a major source of 15:0 in sediments as well. The location of the *n*-alkanes and the C_{18} PUFA suggests different sources for these terrestrial markers. 20:4 ω 6 is close to 18:2 ω 6 and 18:3 ω 3 suggesting a similar terrestrial contribution to 20:4 ω 6. Total PAH is located near total alkanes indicating a similar pathway of delivery. Triacylglycerol is located nearer to the centre suggesting a multiplicity of sources.

Thermochemolysis [106] and sterol analyses were also performed on other sediment samples from Trinity Bay. In the core data all the terrestrial thermochemolysis markers (total phenolics, total phenolics/total fatty acids and 3,4-dimethoxybenzoic acid methyl ester) consistently grouped together and were

always located near POM. In addition, the marine thermochemolysis marker (total fatty acids: [106]) was always associated with dinosterol in sediment data Σ PCA.

Mayzaud et al. [127] also used an elaboration of PCA: PCA on instrumental variables, where one group of variables is selected to explain variations in the other group. In this way they showed close associations between small particles, characteristic of summer, and 16:4 ω 3, 18:3 ω 3, 18:4 ω 3, 18:5 ω 3, 22:6 ω 3 and 22-dehydrocholesterol, and between medium-sized particles, characteristic of the spring bloom and 16:0, 16:1 ω 7, 16:3 ω 4, 16:4 ω 1 and 24-methylenecholesterol. In another seasonal study, Galois et al. [43] used correspondence analysis to show an association of a high bacterial biomass with spring blooms and with river detritus, especially in winter. In another area strongly affected by riverine inputs Yunker et al. [81] used PCA to show that 24-ethylcholesterol, ethylcholest-5,22-dienol and 24-methylcholesterol were terrigenously derived.

PCA and related techniques have been used at several trophic levels above the base of the food web. Using PCA on macrozooplankton, Jeffries and Lambert [126] found 18:0 characterized riverine zooplankton. Following direct hydrolysis and derivatization of fish eggs, Vogt et al. [129] used PCA on 24 GC peaks to distinguish between cod and haddock and between different stages for each species. Navarro et al. [130] used discriminant analysis of fatty acid data to distinguish sea bass larvae fed different diets and anatomical differences in responses to different diets. Two PCA studies of fatty acids in seals suggested that fatty acid composition of dietary lipids was significantly altered before deposition in blubber fat [131] complicating the determination of dietary influences, but that it was possible to distinguish different populations using jaw bones [132]. However, multivariate analysis of a much larger data set [133] has shown that fatty acid signatures of prey are, in fact, reflected in blubber fat.

While chemometrics have been successfully applied to marine food web compartments ranging from dissolved matter to seals an important next step is to use multivariate analyses of biomarkers to objectively define and then quantify trophic relationships in marine ecosystems.

9 Conclusions

Lipid biomarkers are being incorporated to a greater and greater extent in ecological studies; however their use in combination with other techniques greatly strengthens their value. At a first level, the use of individual compounds with synoptic class information or else individual compounds from different classes greatly enhances confidence in source identification. Use of lipids in conjunction with non-lipid markers or stable isotopes can bring a completely new dimension to the picture, especially if multivariate analysis is used. In ecological studies, multivariate analyses are particularly strong when biological data are incorporated into the matrix along side molecular data. We used this approach to investigate the natural biological inputs to Trinity Bay together with the human impacts over the past century. We found that marine compounds enter the food web in a very strong seasonal cycle with maximum inputs occurring in

spring. At this time biogenic fluxes are very high, as is the quality in terms of energy content and essential fatty acids. Very little of this material is buried in the sediments suggesting that it is sequestered in the food web and that the lower trophic levels are functioning efficiently. This appears to have also been the case in the past. Taken together, these observations indicate that the decline in groundfish stocks in this area over the past three decades cannot be related to major shifts in the supply of energy or essential fatty acids to the food web. However, the sink for all the marine lipid material sedimenting out of the water column remains to be established, since it is not the sediments and since there has been a serious decline in groundfish stocks in the area. This represents our next challenge in marine ecosystem studies. Simply identifying sources will not be enough: we are now going to need to be able to quantitatively apportion sources, to identify trophic pathways and to quantify sinks.

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10 References

1. Villanueva J, Grimalt JO, Cortijo E, Vidal L, Labeyrie L (1997) *Geochim Cosmochim Acta* 61:4633
2. Guzman-Vega MA, Mello MR (1999) *Amer Assoc Petr Geol Bull* 83:1068
3. Volkman JK, Barrett SM, Blackburn SI, Mansour MP, Sikes EL, Gelin F (1998) *Org Geochem* 29:1163
4. Forget J, Pavillon J-F, Beliaeff B, Bocquene G (1999) *Env Toxicol Chem* 18:912
5. Mora P, Fournier D, Narbonne J-F (1999) *Comp Biochem Physiol C* 122:353
6. Piechotta G, Lacorn M, Lang T, Kammann U, Simat T, Jenke H-S, Steinhart H (1999) *Ecotox Env Safety* 42:50
7. Sturm A, da Silva de Assis HC, Hansen P-D (1999) *Mar Env Res* 47:389
8. Sargent JR, Parkes RJ, Mueller-Harvey I, Henderson RJ (1987) In: Sleight MA (ed) *Microbes in the Sea*. Ellis Horwood, Chichester, pp 119–138
9. Saliot A, Laureillard J, Scribe P, Sicre MA (1991) *Mar Chem* 36:233
10. Conte MH, Eglinton G, Madureira LAS (1995) *Phil Trans R Soc Lond B* 348:169
11. Hutchings JA, Myers RA (1994) *Can J Fish Aquat Sci* 51:2126
12. Gomes MC, Haedrich RL, Villagarcia MG (1995) *Fish Oceanogr* 4:85
13. Folch J, Lees M, Sloane Stanley GH (1957) *J Biol Chem* 226:497
14. Parrish CC (1988) *Mar Chem* 23:17
15. Parrish CC (1999) In: Arts MT, Wainman BC (eds) *Lipids in freshwater ecosystems*. Springer, Berlin Heidelberg New York, pp 4–20
16. Conte MH, Bishop JKB (1988) *Lipids* 23:493
17. Olsen RE, Henderson RJ (1989) *J Exp Mar Biol Ecol* 129:189
18. Bergen BJ, Quinn JG, Parrish CC (2000) *Env Toxicol Chem*: in press
19. Parrish CC, Bodennec G, Gentien P (1992) *J Chromatogr* 607:97
20. Fraser AJ, Taggart CT (1988) *J Chromatogr* 439:404
21. Ohman MD (1997) *J Plankt Res* 19:1235
22. Parrish CC, Ackman RG (1983) *Lipids* 18:563
23. Rao GA, Riley DE, Larkin EC (1985) *Lipids* 20:531
24. Sasaki GC, Capuzzo JM (1984) *Comp Biochem Physiol* 78B:525

25. Fraser AJ, Tocher DR, Sargent JR (1985) *J Exp Mar Biol Ecol* 88:91
26. Parrish CC (1987) *Can J Fish Aquat Sci* 44:722
27. Vanderploeg HA, Gardner WS, Parrish CC, Liebig JL, Cavaletto JF (1992) *Limnol Oceanogr* 37:413
28. Parrish CC, Bodennec G, Gentien P (1996) *J Chromatogr A* 741:91
29. Yang Z, Parrish CC, Helleur RJ (1996) *J Chromatogr Sci* 34:556
30. Fraser AJ (1989) *Can J Fish Aquat Sci* 46:1868
31. Wakeham SG, Lee C, Farrington JW, Gagosian RB (1984) *Deep-Sea Res* 31:509
32. Parrish CC, Wangersky PJ (1987) *Mar Ecol Prog Ser* 35:119
33. Parrish CC, Wangersky PJ (1990) *J Plankt Res* 12:1011
34. Roessler PG (1990) *J Phycol* 26:393
35. Delbeke K, Teklemariam T, de la Cruz E, Sorgeloos P (1995) *Intern J Environ Anal Chem* 58:147
36. Derieux S, Fillaux J, Saliot A (1998) *Org Geochem* 29:1609
37. Goutx M, Gerin C, Bertrand JC (1990) *Org Geochem* 16:1231
38. Gerin C, Goutx M (1993) *J Planar Chromatogr* 6:307
39. Conte MH, Eglinton G (1993) *Deep-Sea Res I* 40:1935
40. Sikes EL, Volkman JK (1993) *Geochim Cosmochim Acta* 57:1883
41. Parrish CC, Wells JS, Yang Z, Dabinett P (1998) *Mar Biol* 133:461
42. Dunstan GA, Volkman JK, Barrett SM, Garland CD (1993) *J Appl Phycol* 5:71
43. Galois R, Richard P, Fricourt B (1996) *Estuar Coast Shelf Sci* 43:335
44. Parrish CC, McKenzie CH, MacDonald BA, Hatfield EA (1995) *Mar Ecol Prog Ser* 129:151
45. Berge J-P, Gouygou J-P, Dubacq J-P, Durand P (1995) *Phytochem* 39:1017
46. Budge SM, Parrish CC (1999) *Phytochem*:52:561
47. Weeks A, Conte MH, Harris RP, Bedo A, Bellan I, Burkill PH, Edwards ES, Harbour DS, Kennedy H, Llewellyn C, Mantoura RFC, Morales CE, Pomroy AJ, Turley CM (1993) *Deep-Sea Res II* 40:347
48. Budge SM, Parrish CC (1999) *Mar Chem*: submitted
49. Ackman RG (1986) In: Hamilton RJ, Rossel JB (eds) *Analysis of Oils and Fats*. Elsevier, London, pp 137–206
50. Budge SM, Parrish CC (1998) *Org Geochem* 29:1547
51. Claustre H, Marty J, Cassiani L, Dagaut J (1988–89) *Mar Microbial Food Webs* 3:51
52. Bodennec G, Arzul G, Erard-Le Denn E, Gentien P (1994) p 17 in *Tests biologiques et chimiques*. Edition de l'IFREMER, Direction Environment et Aménagement Littoral, R. INT. DEL. 94.07
53. Nichols PD, Palmisano AC, Smith GA, White DC (1986) *Phytochem* 25:1649
54. Viso A, Marty J (1993) *Prog Lipid Res* 32:1521
55. Parkes RJ, Taylor J (1983) *Estuarine Coast Shelf Sci* 16:173
56. Caudales R, Wells J M (1991) *Int J Syst Bacteriol* 42:246
57. Harvey HR, Macko SA (1997) *Org Geochem* 26:531
58. Volkman JK, Johns RB, Gillan FT, Perry GJ (1980) *Geochim Cosmochim Acta* 44:1133
59. Wakeham SG, Beier JA (1991) *Deep-Sea Res* 38:S943
60. Haddad RI, Martens CS, Farrington JW (1992) *Org Geochem* 19:205
61. Harvey HR (1994) *Deep-Sea Res* 41:783
62. Wakeham SG (1995) *Deep-Sea Res I* 42:1749
63. Canuel EA, Martens CS (1993) *Org Geochem* 20:563
64. Santos V, Billett DSM, Rice AL, Wolff GA (1994) *Deep-Sea Res* 41:787
65. Colombo JC, Silverberg N, Gearing JN (1997) *Org Geochem* 26:257
66. Napolitano GE, Pollero RJ, Gayoso AM, MacDonald BA, Thompson RJ (1997) *Biochem Syst Ecol* 25:739
67. Ratnayake WM, Ackman RG (1979) *Lipids* 14:795
68. Graeve M, Hagen W, Kattner G (1994) *Deep-Sea Res I* 41:915
69. Albers CS, Kattner G, Hagen W (1996) *Mar Chem* 55:347
70. Hazel JR, Williams EE, Livermore R, Mozingo N (1991) *Lipids* 26:277

71. Parrish CC, Yang Z, Lau A, Thompson, RJ (1996) *Comp Biochem Physiol* 114B:59
72. Wojciechowski ZA (1991) In: Patterson GW, Nes WD (eds) *Physiology and biochemistry of sterols*. AOCS, Il, p 361
73. Jones GJ, Nichols PD, Shaw PM (1994) In: Goodfellow M, O'Donnell AG (eds) *Chemical Methods in Prokaryotic Systematics*. Wiley, Chichester, pp 163–195
74. Laureillard J, Saliot A (1993) *Mar Chem* 43:247
75. Volkman JK (1986) *Org Geochem* 9:83
76. Patterson GW (1991) In: Patterson GW, Nes WD (eds) *Physiology and biochemistry of sterols*. AOCS, Il, p 118
77. Teshima S (1991) In: Patterson GW, Nes WD (eds) *Physiology and biochemistry of sterols*. AOCS, Il, p 229
78. Idler DR, Wiseman P (1971) *Int J Biochem* 2:516
79. Barrett SM, Volkman JK, Dunstan GA (1995) *J Phycol* 31:360
80. Quemeneur M, Marty Y (1992) *Estuar Coast Shelf Sci* 34:347
81. Yunker MB, Macdonald RW, Veltkamp DJ, Cretney WJ (1995) *Mar Chem* 49:1
82. Colombo JC, Silverberg N, Gearing JN (1996) *Org Geochem* 25:211
83. Parrish CC (1998) *Org Geochem* 29:1531
84. Saliot A (1981) Natural hydrocarbons in sea water. In: Dursuma and Dawson (eds) *Marine organic chemistry: evolution, composition, interactions and chemistry of organic matter in sea water*. Elsevier, New York
85. Bouloubassi I, Saliot A (1991) *Fres J Anal Chem* 339:765
86. Barrick RC, Hedges JI, Perterson ML (1980) *Org Geochem* 21:611
87. Farrington JW, Tripp BW (1977) *Geochim Cosmochim Acta* 41:1627
88. Colombo JC, Pelletier E, Brochu C, Khalil M (1989) *Environ Sci Techn* 23:888
89. Bouloubassi I, Saliot A (1993) *Oceanologica Acta* 16:145
90. Lipiatou E, Saliot A (1991) *Mar Pollut Bulletin* 22:297
91. Jones PW, Leber P (Eds) (1978) *Polynuclear aromatic hydrocarbons*. Ann Arbor Sci Michigan. 892 p
92. Favaro YL (1998) M.Sc. thesis, Memorial University of Newfoundland
93. O'Malley VP (1994) PhD thesis, Memorial University of Newfoundland
94. Favaro YL, Abrajano TA Jr, Helleur RJ (1996) *Proc Biennial International Conference on Chemical Measurement and Monitoring of the Environment*. Ottawa, Canada, May 1996.
95. Challinor JM (1989) *J Anal Appl Pyrolysis* 16:323
96. de Leeuw JW, Baas M (1993) *J Anal Appl Pyrolysis* 26:175
97. Challinor JM (1991) *J Anal Appl Pyrolysis* 29:223
98. Clifford DJ, Carson DM, McKinney JM, Hatcher PG (1995) *Org Geochem* 23:169
99. McKinney DE, Carson DM, Clifford DJ, Minard RD, Hatcher PG (1995) *J Anal Appl Pyrolysis* 34:41
100. Martin F, del Rio JC, Gonzalez-Vila FJ, Verdejo T (1995) *J Anal Appl Pyrolysis* 31:75
101. del Rio JC, Gonzalez-Vila FJ, Martin F, Verdejo T (1994) *Org Geochem* 22:885
102. Pulchan J, Abrajano TA, Helleur R (1997) *J Anal Appl Pyrolysis* 42:135
103. Hatcher PG, Nanny MA, Minard RD, Dible DM, Carson DM (1995) *Org Geochem* 23:881
104. Tanczos I, Schoflinger M, Balla J (1997) *J Anal Appl Pyrolysis* 42:21
105. Tanczos I, Rendl K, Schmidt H (1999) *J Anal Appl Pyrolysis* 49:319
106. Pulchan K (2000) Ph.D thesis, Memorial University of Newfoundland, in preparation.
107. Susic M, Alongi D (1997) *J Chromatogr* 758:243
108. Jun-Kai D, Wei J, Tian-Zhi Z, Ming S, Xio-Guang Y, Chui-Chang F (1997) *J Anal Appl Pyrolysis* 42:1
109. Hayes JM, Freeman KH, Popp BN, Hoham CH (1989) *Org Geochem* 16:1115
110. Kohnen MEL, Schouten S, Sinninghe Damsté JS, de Leeuw JW, Merritt DA, Hayes JM (1992) *Science* 256:358
111. Schoell M, McCafferty MA, Fago FJ, Moldowan JM (1992) *Geochim Cosmochim Acta* 56:1391
112. Schoell M, Schouten S, Sinninghe Damsté J S, de Leeuw J W, Summons RE (1994) *Science* 263:1122

113. Bieger T, Abrajano TA, Hellou J (1997) *Org Geochem* 26:207
114. Abrajano TA, Murphy DE, Fang J, Comet P, Brooks JM (1994) *Org Geochem* 21:611
115. Monson KD, Hayes JM (1980) *J Biol Chem* 255:11435
116. Ostrom NE, Macko S, Deibel D, Thompson R (1997) *Geochim Cosmochim Acta* 61:2929
117. Ramos C (2000) Ph.D thesis, University of the Philippines, in preparation
118. Fang J, Abrajano TA, Comet PA, Brooks JM, Sassen R, MacDonald IA (1993) *Chem Geol* 109:271
119. Laws EA, Popp BN, Bidigare RR, Kennicutt MC, Macko SA (1995) *Geochim Cosmochim Acta* 59:1131
120. Schonkopf S (1999) *American Laboratory* April 1999. p 32
121. Adams MJ (1992) In: Haswell SJ (ed) *Practical guide to chemometrics*. Marcel Dekker, New York, p 181
122. Parrish CC (1987) *Mar Ecol Prog Ser* 35:129
123. Colombo JC, Silverberg N, Gearing JN (1996) *Mar Chem* 51:277
124. Dunteman GH (1989) *Principal Components Analysis*. Sage Publications, Newbury Park, California, p 96
125. Jeffries HP (1979) *Am. Nat.*, 113:643 – 658
126. Jeffries HP, Lambert RM (1982) pp 91 – 101 in *Estuarine Comparisons*, V.S. Kennedy (ed.) Academic Press Inc., New York.
127. Mayzaud P, Chanut JP, Ackman RG (1989) *Mar Ecol Prog Ser* 56:189
128. Dunstan GA, Volkman JK, Barrett SM, Leroi J-M, Jeffrey SW (1994) *Phytochem* 35:155
129. Vogt, NB, Moksness, E, Sporstol SP, Knutsen H, Nordenson S, Kolset K (1986) *Mar Biol* 92:173
130. Navarro JC, McEvoy LA, Amat F, Sargent JR (1995) *Mar Biol* 124:177
131. Grahl-Nielsen O, Mjaavatten O (1991) *Mar Biol* 110:59
132. Grahl-Nielsen O, Mjaavatten O, Tvedt E (1993) *Can J Fish Aquat Sci* 50:1400
133. Iverson SJ, Frost KJ, Lowry LF (1997) *Mar Ecol Prog Ser* 151:255