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Review Article

Lipids in Marine Ecosystems

Christopher C. Parrish

Department of Ocean Sciences, Memorial University of Newfoundland, St. John's, NL, Canada A1C 5S7

Correspondence should be addressed to Christopher C. Parrish; cparrish@mun.ca

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Lipids provide the densest form of energy in marine ecosystems. They are also a solvent and absorption carrier for organic contaminants and thus can be drivers of pollutant bioaccumulation. Among the lipids, certain essential fatty acids and sterols are considered to be important determinants of ecosystem health and stability. Fatty acids and sterols are also susceptible to oxidative damage leading to cytotoxicity and a decrease in membrane fluidity. The physical characteristics of biological membranes can be defended from the influence of changing temperature, pressure, or lipid peroxidation by altering the fatty acid and sterol composition of the lipid bilayer. Marine lipids are also a valuable tool to measure inputs, cycling, and loss of materials. Their heterogeneous nature makes them versatile biomarkers that are widely used in marine trophic studies, often with the help of multivariate statistics, to delineate carbon cycling and transfer of materials. Principal components analysis has a strong following as it permits data reduction and an objective interpretation of results, but several more sophisticated multivariate analyses which are more quantitative are emerging too. Integrating stable isotope and lipid data can facilitate the interpretation of both data sets and can provide a quantitative estimate of transfer across trophic levels.

1. Introduction

This paper concerns compounds that can be operationally defined as lipids. The basis of this definition is their extractability in nonpolar organic solvents which provides a convenient means of separating them from other compounds in an aqueous matrix. These extracts may contain multiple subclasses of both biogenic and anthropogenic origin (Figure 1). The heterogeneous nature of lipids means that they are widely used in ecological and biogeochemical studies assessing the health of ecosystems and the degree to which they have been influenced by terrestrial and anthropogenic inputs. They can be used to determine production of biogenic material of dietary value to marine organisms as well as to indicate water quality. While the emphasis here will be on biochemical and ecological aspects of marine lipids, the more stable compounds have also been extensively used in paleoceanographic studies. Lipid marker determination in sediment cores can show the sensitivity of sediments to changes in land use patterns near land margins. The relationship between aquatic and terrestrially derived products in cores can be used to indicate the degree to which human land use has impacted the pattern of aquatic biogenic productivity in the area.

2. Lipid Analyses

The analysis of lipids starts with sampling design and optimization, if possible, using chemometric procedures [1]. If seawater is being sampled, the next step normally involves the separation into operationally defined dissolved and particulate factions. Kepkay [2] gives the particulate fraction as that retained by filters with a nominal pore size of 0.5–0.7 μm and then gives the colloidal fraction as ranging from 1 nm to 3 nm equivalent spherical diameter at the lower end to 0.2–1.0 μm at the upper end. Samples are then collected and lipids are isolated from the sample matrix usually with chloroform in the case of colloids and truly dissolved material [3], and with mixtures of chloroform and methanol in the case of solids [4, 5]. At this point total lipids, lipid classes, and individual compounds may be measured. Total lipids can be determined gravimetrically, colorimetrically, or by summing individually determined lipid classes which have invariably been separated by chromatography [4].

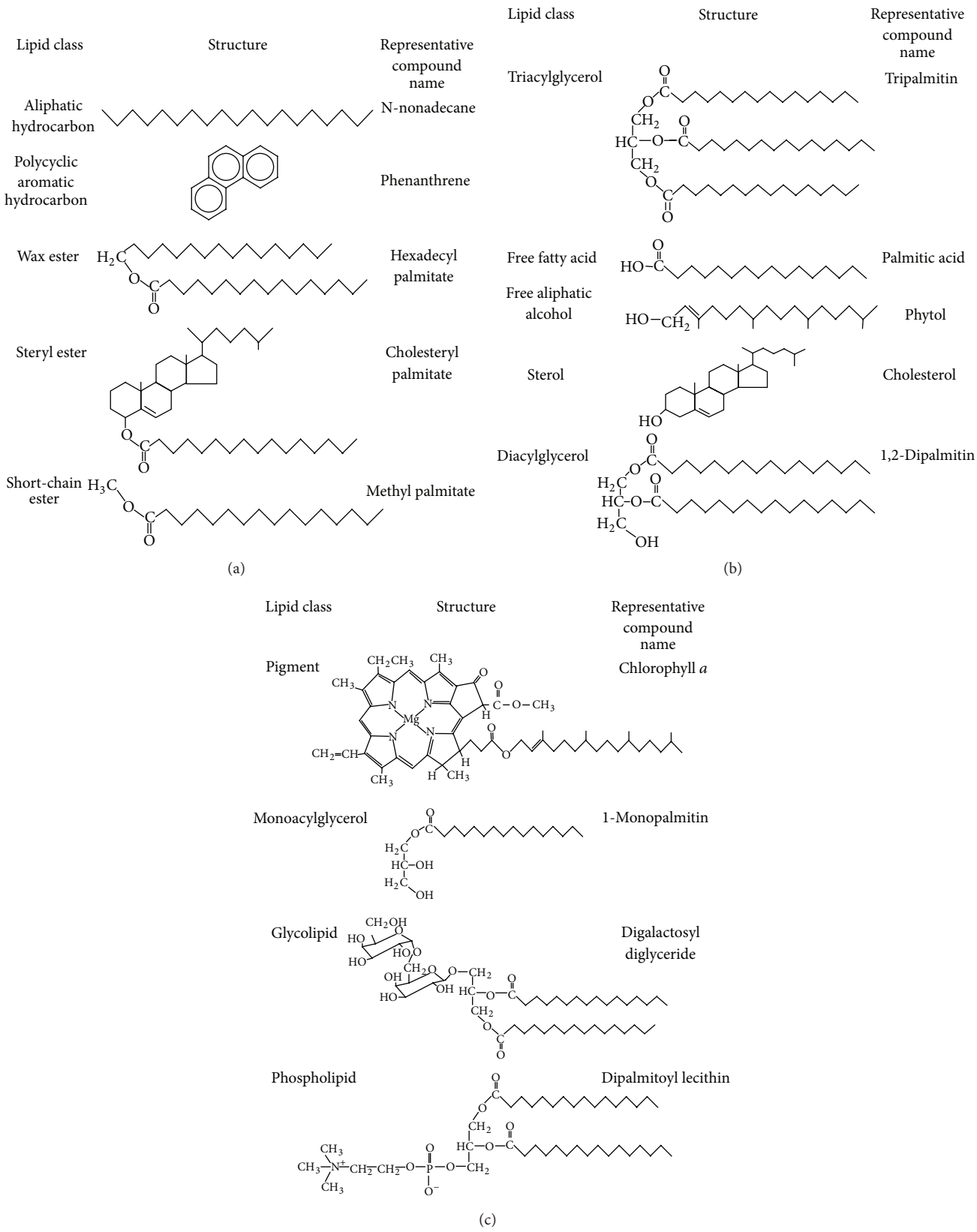


Figure 1: The principal lipid classes of marine samples. The compounds are subdivided into three groups corresponding to class separations that have been obtained with silica gel thin-layer chromatography on Chromarods (Figure 2; [9]). Each compound structure is drawn with the most hydrophobic part of the molecule pointing towards the right of the figure. (a) The least polar classes: the hydrocarbons and simple esters. Wax esters and steryl esters are difficult to separate on Chromarods. (b) Esters, acids, and alcohols. (c) The most polar and most complex classes: the pigments, monoacylglycerols, glycolipids, and phospholipids.

Chromarod thin-layer chromatography (TLC) with Iatroscan flame ionization detection (FID) is used routinely in the quantitative analysis of lipids from aquatic samples (Figure 2). TLC/FID provides synoptic lipid class information and, through summing of classes, a measure of total lipids. The Iatroscan technique has been evaluated against quality assurance standards (QA) originally developed for gas chromatographic analysis at the U.S. Environmental Protection Agency (U.S. EPA, Atlantic Ecology Division). It met the QA criteria prescribed for consistent external calibrations, low blanks, and precise replicate analysis. Relative standard deviations or coefficients of variation (CV) are ~10% [6, 7] and Iatroscan values for aquatic samples are routinely ~90% of those obtained by gravimetry and other methods [6–8]. Gravimetric values tend to be higher, probably because the Iatroscan will determine only non-volatile lipids and because there is always the possibility of the inclusion of nonlipid material in gravimetric determinations.

In Chromarod thin-layer chromatographic separations of lipids from microorganisms, the glycolipids are often eluted in acetone with monoacylglycerols and pigments in a group for which the term "acetone mobile polar lipid" (AMPL) was coined [9]. Being a group, this could present a quantification problem as chlorophyll *a* and the glycoglycerolipids monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) have quite different responses in the Iatroscan; however, the AMPL standard used, 1-monopalmitoyl glycerol (Sigma-Aldrich Co., USA), has a response that is intermediate between them [9].

Capillary gas chromatography (GC) with FID has traditionally been the primary tool used to identify and quantify individual lipid compounds. Other GC detectors that are widely used by organic geochemists are the mass spectrometer (MS) and the electron capture detector (ECD) especially for halogenated compounds [10]. Electron impact (EI) mass spectrometry provides structural information and chemical ionization (CI) mass spectrometry is useful for determining the molecular weight of the organic compound. The high mass resolution capability and mass accuracy of advanced MS instruments has led to their use for halogenated compounds as well [11]. A special type of MS, an isotope ratio mass spectrometer (IRMS), can also be coupled with a GC for compound specific stable isotope analyses (CSIA). To determine isotope ratios in individual compounds the GC is interfaced directly to an IRMS via an in-line combustion oven and a gas purification system [10]. GC-combustion-IRMS is used to characterize the origin and fate of compounds, their breakdown products, and degradation rates in different environments and almost all studies have focussed on determination of isotopes of C ($^{13}C/^{12}C$) and H ($^{2}H/^{1}H$), but new methodologies have been developed to measure isotopes of Cl and Br [12]. High performance liquid chromatography (HPLC) is also used to separate organic compounds, especially pigments and it also can be coupled with mass spectrometry [10], and Thermo Scientific now provides an interface connecting HPLC with IRMS.

Gas chromatography is the most common method for analyzing sterol content and composition and it has been the method of choice in fatty acid analysis for over half a century. Before analysis, however, these compounds must be made more amenable to gas chromatography by derivatization. Sterols are usually analyzed as either trimethylsilyl (TMS) ethers or as sterol acetates, in order to improve their volatility, peak shape, and response factors. Similarly, fatty acids are released from acyl lipid classes and reesterified to methyl esters which provides better separations on GC columns (Figure 3). An acidic (HCl, H_2SO_4 or BF_3) or alkaline ($NaOCH_3$, KOH or NaOH) catalyst is needed in the preparation of fatty acid methyl esters (FAMEs). Because of the variety of derivatization procedures that are described in the literature it is important to take precautions at every step involved and to verify FAME formation and subsequent recovery.

Schlechtriem et al. [13] performed an intercomparison of derivatization procedures for the determination of fatty acids on a sample of salmon flesh and a copepod oil sample. They concluded that the commonly used methanolic BF_3 catalysis was unsuitable for the derivatization of their two samples. BF_3 catalysis is known to be sensitive to moisture and the catalyst should not be used in too high concentrations. The efficiency of a selected catalyst and procedure should be verified (e.g., by thin-layer chromatography) to ensure the complete transmethylation of fatty acids. Once derivatized, fatty acids can be identified by comparison with qualitative standards, for example, Supelco's 37 component FAME mix (Product number 47885-U) and quantitation can be confirmed with a certified standard such as Nu-Chek Prep, Inc.'s GLC Reference Standards (e.g., product no. GLC490). Table 1 provides a listing of fatty acid methyl esters that can be separated on a GC column with a polyethylene glycol phase. Quantitatively, relative standard deviations or CVs should be <5% for repeated analyses.

3. Lipid Nutrition

Aquatic ecosystems occupy the largest part of the biosphere, and lipids in those systems provide the densest form of energy yielding at least two-thirds more energy per gram than proteins or carbohydrates. They are highly reduced compounds and are thus important fuels for oxidation. Lipid energy is transferred from algae to vertebrates via zooplankton and total lipid energy in kilojoules is a predictor of reproductive potential in fish stocks [14]. Marshall et al. [15] found a highly significant linear relationship between total egg production in cod and total lipid energy. Lipids are also a solvent and absorption carrier for fat-soluble vitamins (A, D, E, and K), carotenoids, and organic contaminants. As such, lipid dynamics can be the main drivers of pollutant bioaccumulation in marine ecosystems [16]; thus, the study of lipid flow among trophic levels is important for models of both population dynamics and of bioaccumulation of hydrophobic chemicals.

Among the lipids, certain essential fatty acids (EFAs) and sterols are considered to be important drivers of ecosystem health and stability [18, 19], but despite years of research, they remain among the least well-understood nutrients for aquatic animals [20–24]. EFAs and sterols are essential nutrients in marine bivalves and crustaceans [23, 24] while EFAs

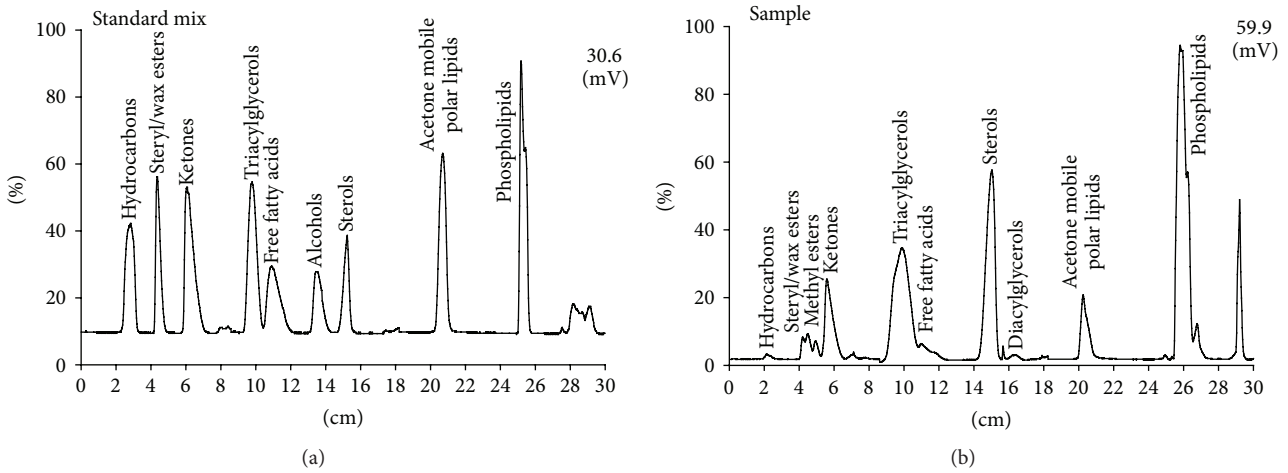


Figure 2: TLC/FID chromatograms of lipid classes in a standard and as part of a study of the effects of produced water from oil and gas production on cod [17]. Lipid class composition was determined after a three-step development on Chromarods (Figure 1). Lipid extracts were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane : diethyl ether : formic acid (98.95 : 1.0 : 0.05). The rods were developed for 25 min, removed from the solvent system for 5 min, and replaced for 20 min and then partially scanned in the Iatroscan. The second development was for 40 min in hexane : diethyl ether : formic acid (79 : 20 : 1) after which the rods were partially scanned. The final separation had two steps, the first was development in 100% acetone for two 15 min time periods, then two 10 min periods in chloroform : methanol : chloroform-extracted water (5 : 4 : 1), after which the rods were dried and their entire length scanned. Before using each solvent system the rods were placed in a constant humidity chamber. The Chromarods were calibrated using standards (e.g., Figure 1) from Sigma Chemicals (Sigma Chemicals, St. Louis, MI, USA).

are also required by echinoderms and vertebrates [24]. The activity of an essential nutrient is expressed physiologically (e.g., in terms of growth response) and biochemically (e.g., in terms of its tissue levels or levels of its metabolites). However, the importance of EFAs stems not only from their impact on animal growth, but also from many other facets of animal function, including reproduction, immunity, and ion balance regulation [22], and even buoyancy control [25]. Part of the lack of understanding of EFAs relates to whether they are essential nutrients or essential metabolites and to whether they are conditionally indispensable or conditionally dispensable [24].

There are two related families of EFAs consisting of ω3 (Figure 4) and ω6 (Figure 5) polyunsaturated fatty acids (PUFA). Fatty acids in each of the families are interconvertible usually through alternate use of desaturases and elongases. The extent to which a given species at a given life stage can convert one ω3 fatty acid to another or one ω6 fatty acid to another determines the degree of essentiality of the fatty acid for that species at that life stage. It should be noted that there are pathways of PUFA synthesis which do not require desaturation and elongation of saturated fatty acids. Polyketide synthase systems conduct the same reactions but in an abbreviated sequence [26]. It has been speculated that PUFA originally synthesized by the polyketide synthase pathway may be a significant contributor to PUFA in marine fish [26].

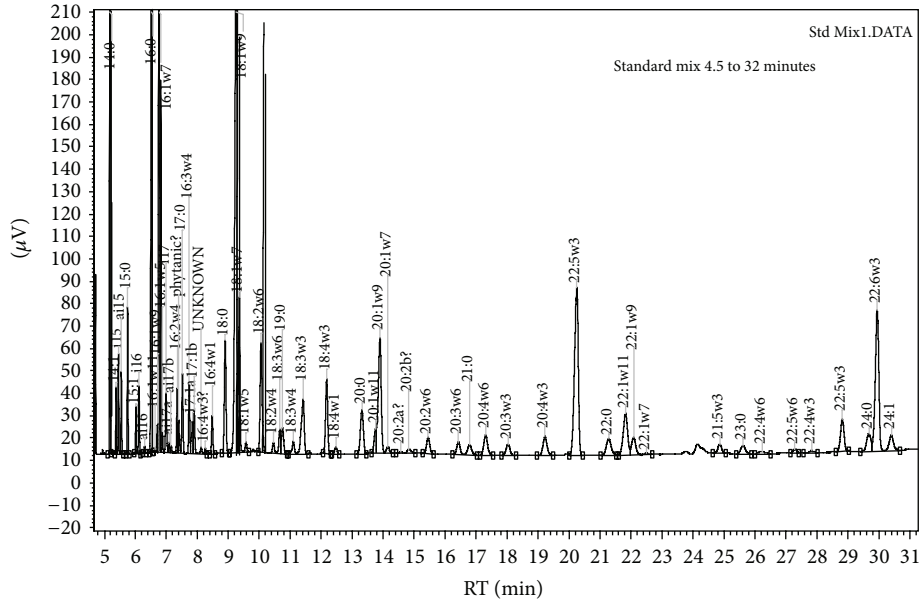
In marine fauna, the focus of EFA work has often been on EPA (eicosapentaenoic acid, 20:5ω3) and DHA (docosahexaenoic acid, 22:6ω3) which have very important functions at various trophic levels [27–29]. For example, the physical properties of EPA in wax esters are thought to be a key factor in buoyancy control in diapausing calanoid copepods [25]. Further up the food web, EPA and DHA are important in stress resistance [30] and immunity [31] in finfish, and dietary DHA is also needed for finfish schooling behaviour [32] and growth in shellfish [29]. DHA from the marine food web is also thought to have played a critical role in human evolution [33].

While EPA and DHA are often quantitatively dominant, there are a number of ω6 long-chain PUFA commonly found in lipid extracts from aquatic food webs, which may be nutritionally important too. ARA (arachidonic acid, 20:4ω6) is one example whose essentiality has often been overlooked [34]. ARA enters several metabolic pathways in finfish [34] and invertebrates, including echinoderms [35] which have high ARA levels [36].

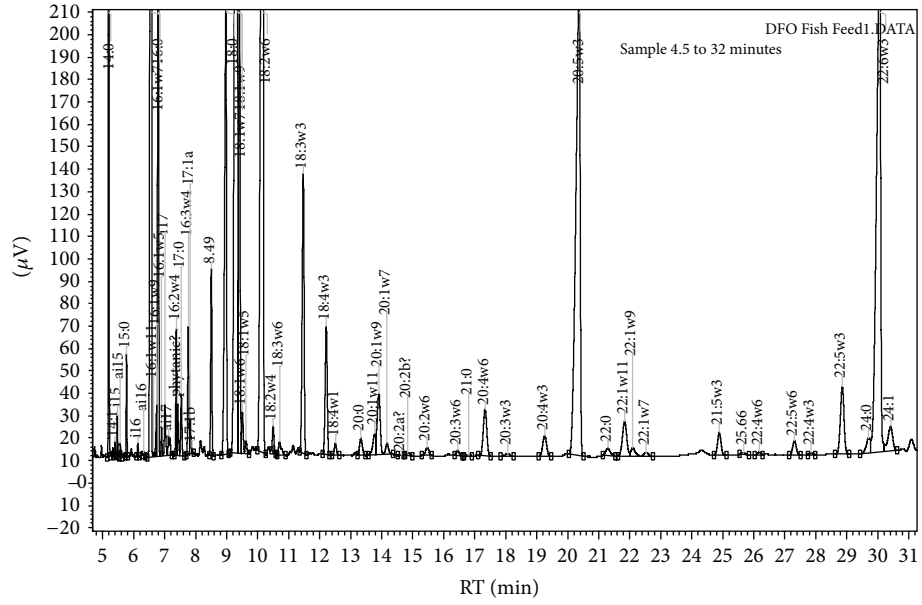
In addition to ARA, there is now another ω6 long-chain PUFA which may be added to the list: ω6DPA (docosapentaenoic acid, 22:5ω6). Of particular interest is the finding of extensive bioaccumulation of ω6 docosapentaenoic acid linked to improved growth in scallop species [37–39] and cod [40] during early ontogeny, suggesting that this is a fourth PUFA which may be essential in marine fauna. ω6DPA may play an important structural role in membranes and/or may be a precursor of bioactive docosanoids. The C_{22} DHA has been found to be a precursor of bioactive compounds generated via enzymatic oxygenations in mammals [41] and trout [42]. The same enzymes could work on the C_{22} ω6DPA to form a parallel series of competitive products as found with the C_{20} EPA and ARA.

4. Lipid Oxidation

There is an increasing interest in oxidative stress in marine ecosystems in the context of global warming and ozone depletion [43]. Lipids are one class of compounds that are



(a)



(b)

Figure 3: GC chromatograms of fatty acid methyl esters in a standard and as part of a study of the effects of produced water from oil and gas production on cod [17]. FAME were analysed on an HP 6890 GC FID equipped with a 7683 autosampler. The GC column was a ZB wax+ (Phenomenex, U.S.A.). The column length was 30 m with an internal diameter of 0.32 mm. The column temperature began at 65°C where it was held for 0.5 minutes. The temperature was ramped to 195°C at a rate of 40°C/min, where it was held for 15 minutes and then ramped to a final temperature of 220°C at a rate of 2°C/min. This final temperature was held for 0.75 minutes. The carrier gas was hydrogen flowing at a rate of 2 ml/minute. The injector temperature started at 150°C and ramped to a final temperature of 250°C at a rate of 120°C/minute. The detector temperature stayed constant at 260°C. Peaks were identified using retention times from a mixture of standards purchased from Supelco, 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033), and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2. A quantitative standard purchased from Nu-Chek Prep, Inc. (product number GLC490) was used to check the GC column performance about every 300 samples (or once a month).

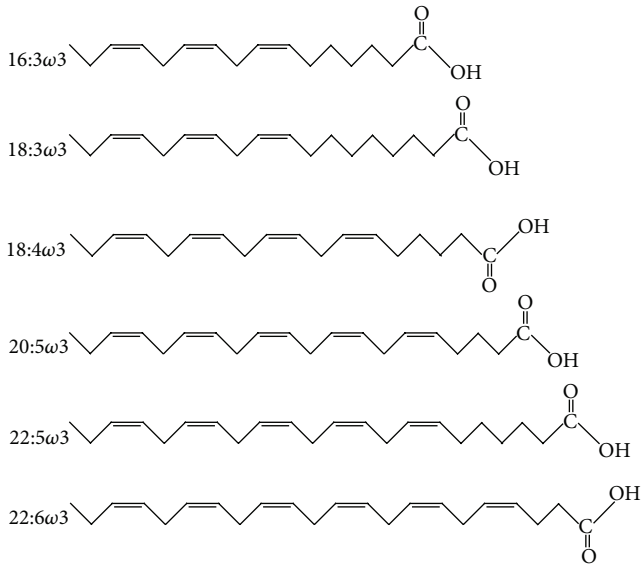


Figure 4: Structures of some $\omega 3$ polyunsaturated fatty acids present in marine samples. Hexadecatrienoic acid (16:3ω3), α-linolenic acid (ALA, 18:3ω3), octadecatetraenoic acid (OTA, 18:4ω3), eicosapentaenoic acid (EPA, 20:5ω3), $\omega 3$ docosapentaenoic acid (ω3DPA, 22:5ω3), and docosahexaenoic acid (DHA, 22:6ω3) are all related biochemically because of the location of the first double bond 3 carbons from the methyl end of the chain.

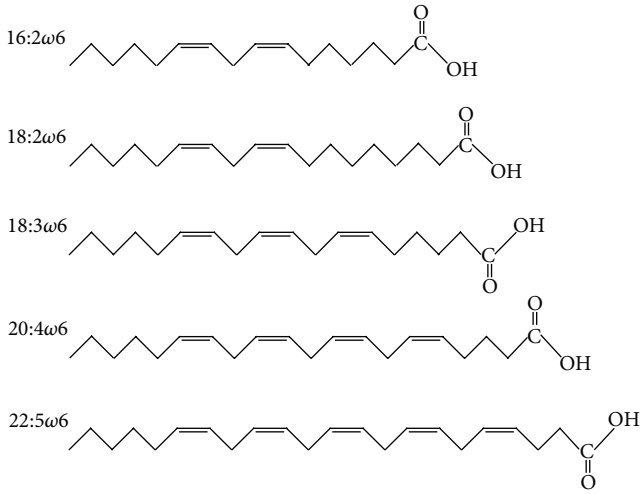


Figure 5: Structures of some $\omega 6$ polyunsaturated fatty acids present in marine samples. Hexadecadienoic acid (16:2ω6), linoleic acid (LA, 18:2ω6), γ-linolenic acid (GLA, 18:3ω6), arachidonic acid (ARA, 20:4ω6), and $\omega 6$ docosapentaenoic acid (ω6DPA, 22:5ω6) are all related biochemically because of the location of the first double bond 6 carbons from the methyl end of the chain.

susceptible to damage leading to a decrease in fluidity (see below) and cytotoxicity [44]. The rate of lipid oxidation depends on several factors such as temperature, presence of inhibitors, catalysts, and the nature of the substrate including the unsaturation level of fatty acids.

Reactions with membranes are one of the most prevalent mechanisms of cellular injury and comparisons in warm and cold environments are particularly interesting because of differences in oxygen solubility and conductance in tissues [43] as well as in membrane unsaturation and rates of reactive oxygen species production and lipid peroxidation [45]. Unsaturated fatty acid oxidative products, oxylipins [46], are cytotoxic for bacteria, fungi, algae, molluscs, crustaceans, and echinoderms [47], suppressing growth and reproduction [48–50]. Oxylipin production in diatoms is wound-activated as would occur during predation and affects population dynamics of copepods [50].

Unsaturated fatty acids have also been implicated in toxicity towards finfish and mammalian cells [51, 52] causing haemolysis [48, 53] and ichthyotoxicity [44]. Toxic fatty acids occur in glycolipids [53, 54] or in the free form [48, 49, 53, 55]. The toxicity of free fatty acids raises some interesting physical chemical questions because of their low solubility in water (<0.1 g/L for C_{12}–C_{15} saturates and <10 mg/L for C_{16}–C_{18} saturates: [56]). However, carboxylic acids are soluble in cold dilute aqueous sodium hydroxide or aqueous sodium bicarbonate, so perhaps the basic nature of seawater enhances the solubility of free fatty acids, and perhaps soap formation ($RCOO^-\ Na^+$) is also a factor in toxicity.

The fact that unsaturated glycolipids have been examined for their toxicity and have also been examined as a source of health promoting omega-3 fatty acids [57] may at first seem contradictory, but the stimulatory and cytotoxic effects of PUFA and their oxidation products are concentration dependent [58]. The toxicity of marine polyunsaturated fatty acids is mediated through oxidation products: polyunsaturated free fatty acids and the superoxide anion (O_2^-) causing destruction of gill cells [44], while PUFA and lipoxygenase in the diatom *Pseudo-nitzschia multiseries* (previously called *Nitzschia pungens*) produce bioactive bacillariolides [59, 60]. Lipoxygenases are enzymes that catalyze the conversion of PUFA to fatty acid hydroperoxides (ROOH). Normally these enzymes require the fatty acids to contain at least two *cis* double bonds interrupted by a methylene group (–CH=CH–CH_2–CH=CHR).

Some fatty acids, or their derivatives, are thought to work synergistically with other known toxins such as domoic acid [59, 61], diarrhetic shellfish poison (DSP) [55], or the neurotoxin brevetoxin [62]. Interestingly, de Boer et al. [62] found that two essential fatty acids the C_{20} PUFA, ARA and EPA were more toxic than the C_{18} PUFA octadecatetraenoic acid (OTA, 18:4ω3). Since ARA and OTA have the same number of double bonds, and ARA was more toxic than EPA which has more, the degree of unsaturation does not seem to be a key determinant of toxicity level.

Sterols are also a source of oxygenated lipids: the oxysterols which are sterols bearing a second oxygen function. These compounds have a variety of biological properties including cytotoxicity and effects on specific enzymes [63]. Cholesterol oxidation products have been linked to yolk sac fry mortality in Atlantic salmon which are depleted in antioxidants [64].

5. Membrane Fluidity

The lipid bilayer in biological membranes forms a permeability barrier for cells and subcellular organelles, and its

Table 1: Fatty acid designations, names, molar masses, and some recent uses as biomarkers. Fatty acid notation gives the ratio of carbon atoms to double bonds. The elution order is that for a GC column with a polyethylene glycol phase. Trivial names are given for most unique fatty acid isomers; otherwise, their systematic names are given. Molar masses are given for fatty acid methyl esters which are useful for the calculation of mol% composition required by some journals and necessary for the calculation of mean chain length, double bond number, and molecular weight [80]. Biomarker references are taken from the past decade.

	Fatty acid name	Molar mass of FAME	Biomarker for	Recent references
14:0	Myristic	242.40	Proteobacteria (*Moritella*)/Diatoms/Prymnesiophytes	[81, 82]
TMTD	Trimethyltridecanoic	270.45		
14:1	Tetradecenoic	240.39	Proteobacteria (*Colwellia, Moritella*)	[81]
*i*15:0	Methyltetradecanoic	256.43	Bacteria	[83]
*ai*15:0	Methyltetradecanoic	256.43	Bacteria	[83]
15:0	Pentadecanoic	256.43	Phytoplankton	[84]
15:1	Pentadecenoic	254.41	Bacteria	[85]
*i*16:0	Methylpentadecanoic	270.45		
*ai*16:0	Methylpentadecanoic	270.45	Bacteria	[85]
Pristanic	Tetramethylpentadecanoic	312.53		
16:0	Palmitic	270.45		
16:1ω11	Hexadecenoic	268.43		
16:1ω9	Hexadecenoic	268.43		
16:1ω7	Palmitoleic	268.43	Mangrove/Diatoms/Bacteria	[83]
16:1ω 5	Hexadecenoic	268.43	Bacteria	[83]
*i*17:0	Methylhexadecanoic	284.48	Bacteria	[83]
*ai*17:0	Methylhexadecanoic	284.48	Bacteria	[83]
16:2ω4	Hexadecadienoic	266.42	Bacteria	[83]
Phytanic	Tetramethylhexadecanoic	326.55		
17:0	Margaric	284.48	Bacteria	[82]
16:3ω4	Hexadecatrienoic	264.39		
17:1	Heptadecenoic	282.46	Bacteria	[84]
16:4ω3	Hexadecatetraenoic	262.39		
16:4ω1	Hexadecatetraenoic	262.39	Diatoms	[82]
18:0	Stearic	298.51		
18:1ω11	Octadecenoic	296.49		
18:1ω9	Oleic	296.49	Deep-sea fish/Crustaceans/Macroalgae/Mangrove/Carnivory	[82, 83]
18:1ω7	Vaccenic	296.49	Bacteria	[83]
18:1ω6	Octadecenoic	296.49	Bacteria	[83]
18:1ω5	Octadecenoic	296.49		
18:2a	Octadecadienoic	294.48		
18:2b	Octadecadienoic	294.48		
18:2ω6	Linoleic; LA	294.48	Mangrove/Seagrass/Macroalgae/Vascular plants	[83]
18:2ω4	Octadecadienoic	294.48	Bacteria	[83]
18:3ω6	γ-linolenic; GLA	292.46	Macroalgae	[83]
19:0	Nonadecanoic	312.54	(*N.b. can be difficult to separate from 18:3ω6*)	
18:3ω4	Octadecatrienoic	292.46		
18:3ω3	α-linolenic; ALA	292.46	Mangrove/Seagrass/Vascular plants	[83]
18:4ω3	Stearidonic; OTA	290.43	Cryptophytes/Haptophytes/Dinoflagellates/Cryptomonads/Seagrass /Kelp	[83]
18:4ω1	Octadecatetraenoic	290.43		
20:0	Arachidic	326.56		
18:5ω3	Octadecapentaenoic	288.42	Dinoflagellates	[81]

Table 1: Continued.

	Fatty acid name	Molar mass of FAME	Biomarker for	Recent references
20:1ω11	Gadoleic	324.54	Copepods	[83]
20:1ω9	Gondoic	324.54	Copepods	[83]
20:1ω7	Eicosenoic	324.54		
20:2α	Eicosadienoic	322.53		
20:2β	Eicosadienoic	322.53		
20:2ω6	Eicosadienoic	322.53		
20:3ω6	Dihomo-γ-linolenic	320.51		
21:0	Heneicosanoic	340.59		
20:4ω6	Arachidonic; ARA	318.50	Protozoa and microeukaryotes/red algae/Kelp	[83]
20:3ω3	Eicosatrienoic	320.51		
20:4ω3	Eicosatetraenoic	318.50	Fungi/Protozoa/Algae	[83]
20:5ω3	Eicosapentaenoic; EPA	316.48	Diatoms and brown and red macroalgae	[83]
22:0	Behenic	354.61	Terrestrial plants	[86]
22:1ω11(13)	Docosenoic	352.60	Zooplankton	[85]
22:1ω9	Erucic	352.60	Zooplankton	[85]
22:1ω7	Docosenoic	352.60		
22:2NMIDa	Docosadienoic	350.58		
22:2NMIDb	Docosadienoic	350.58		
21:5ω3	Heneicosapentaenoic	330.51		
23:0	Tricosanoic	368.64		
22:4ω6	Adrenic	346.55		
22:5ω6	Osbond; ω6DPA	344.53		
22:4ω3	Docosatetraenoic	346.55		
22:5ω3	Clupanodonic; DPA	344.53		
24:0	Lignoceric	382.66	Mangrove/terrestrial plants	[87]
22:6ω3	Docosahexaenoic; DHA	342.51	Dinoflagellates/zooplankton	[83]
24:1	Tetracosanoic	380.65	Zooplankton	[87]

ai: *anteiso* methyl-branched, DPA: docosapentaenoic acid, *i*: *iso*-methyl branched, NMID: nonmethylene interrupted dienoic, OTA: octadecatetraenoic acid.

physical characteristics are a key determinant of membrane structure and function. The lipid core of a biological membrane must be sufficiently fluid to permit lateral movement of the constituent lipids and embedded proteins such as transmembrane proteins. Ectotherms can counteract the ordering effects of reduced temperature or increased pressure by changing the structure of their membranes [65, 66], a process known as homeoviscous adaptation [67]. This biochemical adaptation involves remodeling membrane lipids: changes in phospholipid to sterol ratios and acyl chain length and saturation have been observed in bivalves and finfish [68, 69]. Hard clams in Atlantic Canada increased the level of unsaturation of fatty acids in their gills throughout the fall and then increased their phospholipid to sterol ratio in December [68].

Although it is generally accepted that ectothermic animals increase membrane content of unsaturated fatty acids in response to cold to maintain fluidity [70], few studies have shown a clear and direct relationship between unsaturated fatty acids and membrane fluidity in marine organisms [67, 71]. While phospholipid molecular species containing 22:6ω3 at the *sn*-2 position are believed to be important in controlling finfish membrane fluidity [72–75], a direct correlation with 22:6ω3 has not been found [72, 73, 75, 76]. In contrast, Hall et al. [71] found a simple but very strong relationship between fluidity and a single PUFA, 20:5ω3, in gill membranes from the sea scallop *Placopecten magellanicus* following a 10°C temperature drop. This indicates that scallops are excellent experimental animals for investigating the link between temperature and nutrition in the natural environment or in culture.

There is another possible mechanism that is available to bivalves that has received little attention. Bivalves are unusual in having a wide array of sterols available for incorporation in membranes [77, 78]. Sterols control membrane fluidity and permeability in eukaryotic organisms, and marine bivalves require a dietary supply for somatic growth [23]. Those living in surface waters may change the chemical composition of sterols in response to seasonally varying temperature, while those living in deep waters may use other sterols in the face

of constantly very cold conditions and higher pressure. The ordering capacity provided by cholesterol is different to that provided by other sterols [23]. Sea scallops living at 10 m and 31 m depths in Newfoundland waters have different sterols in their adductor muscle, especially 24-methylenecholesterol [79], which is a major sterol in cold water bivalves [77]. Whether this has any effect on membrane fluidity, or there is a seasonal pattern, or there is any seasonal correlation with source sterols have yet to be determined.

6. Molecular Biomarkers

Biological markers are receiving widespread attention in ecological, paleoecological, and biogeochemical studies in aquatic environments [10]. Chemical markers are compounds or groups of compounds that can be used as indicators or signatures of individual organisms or of groupings of organisms, or of certain environmental processes. In order to measure inputs, cycling, and loss of materials in marine ecosystems, there are now a wide range of compounds and instrumental tools that are available. Anthropogenic compounds can be used as wastewater markers to locate sources and pathways of transport as well as to determine pollutant loadings. Molecular biomarkers can be DNA fragments or smaller molecules that are easily determined using standard chromatographic separation techniques. Lipids are one such group that are receiving continuing attention in ecological (e.g., [88]) and biogeochemical [89] studies.

The heterogeneous nature of lipids means that much information can be gained by determining individual classes. Individual classes or groups of certain classes may be used to indicate the presence of certain types of organisms as well as their physiological state and activity [90]. Lipid classes can also be used to indicate sources of organic matter [91] including dissolved organic matter and hydrophobic contaminants [89, 92]. Iatroscan-determined free fatty acids, alcohols, diacylglycerols, and monoacylglycerols in filtrates indicated seasonal, day/night, and depth differences in degradation in the North West Mediterranean Sea, while hydrocarbons showed the level of contamination [89, 92].

Lipid class information is particularly valuable when used in conjunction with determination of individual fatty acids [93] or sterols [94] or both [95]. Another combination, fatty acids, *n*-alkanes and glycerol dialkyl glycerol tetraethers (GDGTs) has been used to assess differences in plankton composition and food quality in the Humboldt Current System [86]. GDGTs are found in prokaryote membranes and they contain ether linkages to glycerol (R–O–C–) rather than ester linkages as found in glycerophospholipids (RCOOC–). HPLC-MS analyses of intact GDGTs in sediments also show great promise for identifying the delivery of soil-derived organic matter to coastal regions as well as for paleothermometry [10].

Fatty acids and sterols are versatile biomarkers that are widely employed in oceanographic studies because of the large number of unique structures that are synthesized. Fatty acids have often been broadly used as biomarkers in trophic transfer studies in aquatic food webs (e.g., [96–98]) and now they are being increasingly used for analysing terrestrial food webs as well [99]. Information provided by these markers may be used to delineate carbon cycling and transfer of material through food webs especially when analysed with multivariate statistics (see below).

Using fatty acids, bacteria, phytoplankton classes, and zooplankton orders may be distinguished in marine samples [81, 88, 93]. Table 1 gives a listing of recently used fatty acids and it can be seen that many compounds are not unique biomarkers. When this occurs, fatty acid sums and ratios can be added to help in the interpretation. For example, Pepin et al. [100], employing a variety of markers of diatoms (16:1ω7 + 16:4ω1 + 20:5ω3; 16:1ω7/16:0; $\Sigma C_{16}/\Sigma C_{18}$), prymnesiophytes (18:1$\omega$9 + 18:4$\omega$3), and dinoflagellates (22:6ω3/20:5ω3) showed diapausing copepods in slope waters had fed mainly on diatoms while active ones in shelf waters had fed mainly on dinoflagellates and prymnesiophytes. Another ratio has been used in benthic organisms, the 18:1 isomer ratio, to indicate diatom feeding and trophic level [101]. The rationale behind the use of these ω9/ω7 ratios is elongation of the diatom 16:1 ω7 to 18:1ω7, but they could be biased if there was a significant intake of bacterial 18:1ω7. Low and seasonally highly variable ratios have been observed in echinoderm gonads [102, 103].

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 [81, 88, 104]. For example, Kreibich et al. [105] used 20:1ω9 and 22:1ω11 to show calanoid copepods were a major food source for two pelagic crustaceans in the Greenland Sea.

Sterols and hydrocarbons are also excellent biomarkers due to their stability in addition to the diversity of their structures. They have been extensively used in sedimentary geochemical studies (e.g., [106–108]) and aquatic biogeochemical studies [10]. For example, Hudson et al. [109] grouped sterols in order to apportion inputs to a cold ocean ecosystem and showed the dominance of marine origin C_{27} and C_{28} sterols in plankton and settling particles contrasted with higher plant C_{29} sterols dominating in the underlying sediments. As part of a trophic ecology study, Drazen et al. [95] measured sterols to help assess feeding habits of abyssal echinoderms and used C_{28} and C_{29} sterols, especially 24-methylcholesta-5,22E-dien-3β-ol (brassicasterol) and 24-ethylcholest-5-en-3β-ol (sitosterol), to show the importance of phytodetritus. The use of sterols as trophic markers in the lower food web should be investigated further. Indeed, further sterol studies in modern environments will greatly aid source identification in sediments (e.g., [108]).

7. Multivariate Data Analysis

Application of chemometric methods for experiment design and analysis of multivariate data is becoming increasingly common as it permits data reduction and an objective interpretation of the results. In the marine biomarker field, principal components analysis (PCA) has gained popularity as a powerful data reduction procedure. This multivariate technique handles a large amount of variables simultaneously, and has been used by organic geochemists to determine organic matter sources and degradation of suspended and sinking particles in the oceans [110, 111]. We have used PCA

to determine marine, terrestrial, and anthropogenic organic inputs, and trophic relationships (e.g., [100, 102, 104, 112–114]). Fatty acid PCA continues to have a strong following [115–117], but more sophisticated multivariate statistical analyses are emerging too (see below).

There are several refinements to PCA that have been applied, such as expansion beyond the first two principal components. PC1 accounts for most of the variability in the data set, PC2 the next largest, and so on. When PC1 and PC2 account for most of the overall variation in the data set between them, the data can easily be represented diagrammatically in only two dimensions. For example, PC1 and PC2 in Pepin et al. [100] explained 92% of the variation in copepod *Calanus finmarchicus* fatty acid summary data and there was a clear separation of samples from shelf and slope locations. In most studies, it requires more than two principal components to account for that much of the variation. Xue et al. [111] used three principal components to construct organic matter degradation trajectories as against a one-dimensional degradation index [110]. Xue et al. [111] used cluster analysis to help determine the number of principal components to consider. Even if more than half of the variation in the data set is accounted for by PC1 and PC2, indicating the sign (+ or −) of the coefficients and scores on PC3 can be useful. In a copepod feeding study the first two principal components accounted for 54.2% of the variation and addition of the third principal component raised this value to 73.8% [114]. The co-location of *Calanus* spp. and its diet in three PC dimensions showed that there was little modification or sequestration of dietary polyunsaturated fatty acids. This study also used cluster analysis but this time the scores were grouped by single linkage cluster analysis in order to determine which should be encircled in the graphic representation. Allan et al. [117] used one-way analysis of variance (ANOVA) for the same purpose and showed spatial changes in mussel diets.

An additional refinement is the further analysis of PCA data. PC1 scores have been used to indicate how source composition of suspended and sinking particles varies with depth [110], and how the accumulation of reserves in oysters varies with season [118]. They have also been correlated with lengths of *Calanus finmarchicus* [100] and lengths of beluga whales [115] to reveal size-related dietary differences. Further principal components could be included in the analyses using multiple linear regressions [1].

8. Quantification of Trophic Relationships

Despite several refinements that can be applied, principal components analysis remains essentially a means to provide a simple graphic representation of an entire data set. More sophisticated multivariate statistical analyses can provide a more quantitative analysis of patterns, and such techniques are emerging for use with fatty acids, especially from food web samples [119, 120]. These authors used PRIMER (Plymouth Routines in Multivariate Ecological Research) to investigate algae feeding amphipods and sea urchins. This is a software package for analyzing ecological data marketed by PRIMER Enterprises (PRIMER-E) Ltd. They used analysis of similarities (ANOSIM), a nonparametric approximate analogue of univariate ANOVA tests, to provide a measure of similarity between groups, and similarity of percentages analysis (SIMPER) to examine the contribution of each fatty acid to average resemblances between sample groups. SIMPER identifies that which is primarily providing the discrimination between observed sample clusters.

ANOSIM and SIMPER provide a more quantitative analysis of patterns in the lower food web, but they do not provide quantitative estimates of predator diets as can be done at higher trophic levels [96, 98]. Prey consumption of seals, seabirds, and polar bears has been estimated by statistically comparing predator fatty acid signatures with those of their potential prey.

The quantitative fatty acid signature analysis (QFASA) model [96] is based on minimizing the statistical distance between predator signatures and mean prey signatures. Predator signatures must also be corrected for modification that may occur due to predator fatty acid metabolism using calibration coefficients. Calibration coefficients are developed using long-term controlled diet studies to estimate differences between patterns of fatty acid intake and deposition in predators. The coefficients correspond to the ratio of prey composition to predator tissue composition for each fatty acid, after assuming it has consumed the diet long enough to have completely turned over all fatty acids. Thus for fatty acids to be quantitative determinants of diet, an experimental approach is required to determine selective retention of specific fatty acids, to detect *de novo* synthesis, elongation and/or desaturation, and to measure extents of tissue-specific deposition. It is interesting that this is the same question being asked in the field of aquaculture nutrition in different ontogenetic stages of multiple species from several phyla. For example, dietary and tissue fatty acids and sterols have been measured to determine incorporation efficiencies or enrichments and depletions in yellowtail flounder [121] and sea scallops [37, 78]. Such data may help in the application of QFASA to fish and primary consumers; however, the breadth of species and differences in lipid metabolism in the lower food web may make quantitative estimates of fatty acid flow through the lower food web difficult.

The determination of isotopes of C ($^{13}C/^{12}C$) and N ($^{15}N/^{14}N$) may also help estimate fatty acid flow through the lower food web. Quantitative estimates of ice algae contributions to higher trophic levels have been made using compound-specific stable isotope analyses of fatty acid $^{13}C/^{12}C$ ratios ($\delta^{13}C$ in ‰: [122]). Fatty acid isotopic signatures were also used in a three-step food chain feeding experiment to show that polyunsaturated fatty acids originally synthesized by the polyketide synthase pathway could be a significant contributor to PUFA in marine fish [40]. In addition there are implications for food web $\delta^{13}C$ as fewer synthetic steps will cause less kinetic fractionation of the isotope at the original point of synthesis. This stable isotope mass spectrometer methodology should be investigated further for fatty acid and sterol work.

Bulk $^{15}N/^{14}N$ ratios ($\delta^{15}N$ in ‰) have traditionally been used to determine trophic levels of consumers. Connelly et al. [84] correlated bulk stable isotope ratios (‰) and fatty acid

proportions (% of total fatty acids) in 26 taxa of zooplankton collected from the benthic boundary layer on the Beaufort Sea shelf. For those fatty acids that had a significant correlation with δ^{15}N, a trophic multiplication factor (TMF) was calculated from the slope of the linear regression on δ^{15}N determined trophic level. Docosahexaenoic acid had by far the greatest average increase per trophic level among the PUFA, suggesting that this essential fatty acid was highly conserved through the food web and was not used as an energy source.

Carreón-Palau et al. [123] calculated trophic retention factors (TRFs) for fatty acids in a coral reef food web with river influence in the Gulf of Mexico. These factors were calculated in a manner similar to that used for trophic magnification factors (TMFs) of contaminants. Concentrations of the essential fatty acids 20:5ω3, 20:4ω6, and 22:6ω3 showed positive slopes across trophic levels with the slopes increasing in the order shown, suggesting an order of essentiality. Possible mechanisms for significantly positive TRFs include preferential assimilation of essential fatty acids as has been documented in finfish [124] and preferential oxidation of nonessential fatty acids as again documented in finfish [125]. This regulation by consumers provides an important distinction from trophic magnification of highly lipophilic nonmetabolized contaminants.

A further refinement of the TRF approach is to estimate fatty acid TRFs according to source. Carreón-Palau et al. [123] used bulk δ^{13}C and δ^{15}N to apportion primary producer sources of organic carbon in a coral reef food web. The most probable solution was calculated using an isotopic mixing model: Stable Isotope Analysis in R (SIAR) which is available as an open source R package. R is a free software environment for statistical computing and graphics. Using these data, it was possible to estimate the contribution from each primary producer to each fatty acid in consumers. In turn, these data were used to calculate the trophic retention factors for each fatty acid from each primary producer source.

9. Conclusions

In marine ecosystems, lipids provide the densest form of energy which is transferred from algae to vertebrates via zooplankton. As well, they contain essential fatty acids and sterols which are considered to be important drivers of ecosystem health and stability. Fatty acids and sterols are also susceptible to oxidative damage leading to cytotoxicity and a decrease in membrane fluidity. The physical characteristics of biological membranes can be defended from the influence of changing temperature, pressure, or lipid peroxidation by altering the fatty acid and sterol composition of the lipid bilayer. The influence of essential lipids, lipid oxidation, and membrane composition on food web structure and function will become increasingly important in the context of global warming and ozone depletion.

Lipids have been used as biomarkers in studies ranging from bacteria to bears. Lipid classes and their components, especially fatty acids, have been the main tools used, but the determination of individual intact lipids or molecular species is gaining momentum. The measurement of intact polar lipids such as glycerol dialkyl glycerol tetraethers rather than their subcomponents, such as fatty acids derived from glycerophospholipids, would strengthen the identification of microorganisms. The importance of different fatty acids and phospholipids with different head groups in regulation of cellular processes, together with the fact that fluidity, may be controlled by just a few compounds suggests that molecular species analysis would also help describe mechanisms behind the ecological effects of essential fatty acids.

Data sets of lipids from marine ecosystems are often interpreted using chemometrics as they permit data reduction and an objective interpretation of the results; however, a key step is to use multivariate analyses of biomarkers to objectively define and then quantify trophic relationships in marine ecosystems. This represents our current challenge in ecosystem studies: simply identifying sources is no longer enough. To quantify trophic relationships, multiple markers may be necessary. In this regard, the use of sterols as trophic markers in the lower food web should be investigated further. Sterols are excellent biomarkers due to their stability in addition to the diversity of their structures.

The determination of isotopes of C (^{13}C/^{12}C) and N (^{15}N/^{14}N) may also help estimate lipid flow through the food web. Integrating bulk stable isotope data with fatty acid data can facilitate the interpretation of both data sets and can provide a quantitative estimate of transfer across trophic levels. Compound-specific stable isotope analyses can also differentiate inorganic sources and biosynthetic pathways in primary producers and provide estimates of contributions to higher trophic levels. Application of bulk and compound-specific stable isotope mass spectrometer methodology and its modeling should be investigated further for both fatty acid and sterol work.

Abbreviations and Definitions

$^{A}_{Z}$X: Atoms are symbolized by $^{A}_{Z}$X where A is the mass number in amu, Z is the atomic number and X is the element symbol. Isotopes of an element have the same Z but a different A

A:BωX: Fatty acids are designated with the convenient shorthand notation A:BωX, where A is the number of carbon atoms, B is the number of double bonds and X is the position of the double bond closest to the terminal methyl group (Table 1). With this system, all double bonds are assumed to be methylene-interrupted and *cis* in configuration

AMPL: Acetone-mobile polar lipid

ANOSIM: Analysis of similarities

ARA: Arachidonic acid (20:4ω6)

CSIA: Compound specific stable isotope analyses

$\delta^{13}C_{FA}$: Natural carbon isotope compositions for fatty acid methyl esters are reported in per mille (‰) using the conventional delta notation (δ^{13}C), relative to the Vienna PDB standard: $\delta^{13}\,C_{FA}$ (‰) = [(^{13}C / $^{12}C_{FA}$ / ^{13}C / $^{12}C_{PDB}$) − 1] · 1000

DGDG: Digalactosyl diacylglycerol
DHA: Docosahexaenoic acid (22:6ω3)
ω6DPA: Docosapentaenoic acid (22:5ω6)
CI: Chemical ionization
ECD: Electron capture detector
EFA: Essential fatty acid(s)
EI: Electron impact
EPA: Eicosapentaenoic acid (20:5ω3)
FID: Flame ionization detection
GC: Gas chromatography
GDGTs: Glycerol dialkyl glycerol tetraethers
HPLC: High performance liquid chromatography
IRMS: Isotope ratio mass spectrometer
MGDG: Monogalactosyl diacylglycerol
MS: Mass spectrometer
OTA: Octadecatetraenoic acid (18:4ω3)
PC: Principal component(s)
PRIMER: Plymouth Routines in Multivariate Ecological Research
PUFA: Polyunsaturated fatty acid(s)
QA: Quality-assurance
QFASA: Quantitative fatty acid signature analysis
R: R, derived from radical, represents an alkyl group: C_nH_{2n+1}
SIMPER: Similarity of percentages
sn-2: Stereospecific numbering to indicate the middle position on the glycerol
TLC: Thin-layer chromatography
TMF: Trophic magnification/multiplication factor
TRF: Trophic retention factor.

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