



FEATURE ARTICLE: REVIEW

Fatty acids as dietary tracers in benthic food webs

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ABSTRACT: Fatty acid (FA) analysis is a well-established tool for studying trophic interactions in marine habitats. However, its application to benthic food webs poses 2 particular challenges. First, unlike pelagic zooplankton, benthic consumers have access to different sizes and functional groups of primary producers and may consume a highly mixed diet. Classes of benthic primary producers are distinct in their overall FA composition, but most do not possess unique marker FAs that can be used to identify their contribution to higher trophic levels. Second, unlike mammalian predators, benthic invertebrates have the capacity to significantly modify their dietary FAs and thereby obscure markers for food sources. Controlled feeding studies have been used to distinguish dietary tracer FAs from those that are modified by the consumer in several benthic invertebrates, but more such studies are needed. Despite these challenges, FAs have been used to study trophic structure in a variety of benthic habitats including the deep sea, polar regions, estuaries, and the rocky subtidal zone. However, the complexity of benthic food webs and lack of unique markers impose uncertainties in the interpretation of FA data from field studies. Multivariate analyses are necessary for analyzing FA datasets, although univariate tests can be useful for comparing levels of informative FAs among food sources and consumers. Combining FA analysis with other lines of evidence, such as stable isotope analysis, offers a more reliable approach to examining trophic interactions in benthic systems.

KEY WORDS: Fatty acids · Trophic interactions · Benthic consumers · Macroalgae · Invertebrates

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The diversity of benthic primary producers poses a challenge for identifying the diets of invertebrate grazers using fatty acid analysis.

Photo: Robert Scheibling

INTRODUCTION

Identifying the diets of animals is important for understanding their basic ecology, characterizing trophic interactions, and predicting community-level consequences of biotic and abiotic change. In the marine environment, direct observation of feeding is often impractical or impossible, so ecologists have developed indirect methods for examining diet. Physical methods such as analyzing gut contents or fecal pellets have been used extensively, but these can underestimate the importance of soft and highly digestible food items and overestimate that of recently consumed items (Graeve et al. 2001, Latyshev

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et al. 2004). These methods may also be inappropriate for invertebrates with unusual modes of feeding or digestion; for example, many sea stars feed extra-orally and are rarely observed with any gut contents (Howell et al. 2003). Biochemical tracer methods such as stable isotope or fatty acid (FA) analysis have the advantage of potentially providing less biased, longer-term dietary information (Iverson et al. 2004).

FAs have been used extensively in pelagic food webs, especially to identify phytoplankton and characterize the diets of zooplankton (reviewed by Dalsgaard et al. 2003). Diatoms and dinoflagellates are distinct in their FA composition, and zooplankton tend to retain dietary FAs in their tissues. Calanoid copepods also synthesize a few relatively rare FAs that can be used to identify their contribution to the diets of higher-order consumers (Sargent & Falk-Petersen 1988, Dalsgaard et al. 2003). Top predators have also been successfully studied using FA analyses; dietary FAs are deposited in the blubber of marine mammals with relatively little modification, facilitating accurate characterization of their diets (reviewed by Budge et al. 2006, Iverson 2009).

Unlike pelagic systems, where phytoplankton is the major source of primary production, benthic food webs are supported by a diversity of primary producers that can include macroalgae and vascular plants, in addition to phytoplankton and bacteria (Kharlamenko et al. 2001, Dalsgaard et al. 2003). Although these primary producers have characteristic FA compositions, the larger number of potential food items can pose problems for distinguishing their contributions to diets, particularly when animals consume mixed diets (Kharlamenko et al. 2001, Alfaro et al. 2006). Some invertebrate taxa are capable of modifying dietary FAs such that their FA composition may not directly reflect diet (Kelly et al. 2008, 2009). Most taxa lack a specific fat-storage organ, so other functions of lipid-rich tissues may influence their FA composition to a greater extent than does diet (Hughes et al. 2006). Despite these challenges, many studies have employed FAs to better understand animal diets and trophic relationships in benthic habitats.

In this review, we examine the utility of FAs as bottom-up dietary tracers in benthic marine systems by comparing FA composition among benthic primary producers, summarizing laboratory-based controlled feeding studies, discussing field studies using FAs in various benthic habitats, evaluating statistical analyses for FA

data, and comparing stable isotope and FA analyses as techniques for studying benthic food webs. We do not cover extraction or chemical analysis of FAs and refer readers to Budge et al. (2006) for advice on these processes.

FA NOMENCLATURE AND TERMINOLOGY

FAs consist of a terminal methyl group (CH_3), a carbon chain, and a terminal carboxyl group (COOH). Most naturally-occurring FAs have an even number of carbons, usually between 14 and 24, although vascular plants commonly have long-chain FAs of up to 28 carbons in their cuticular waxes (Gurr et al. 2002). Saturated FAs (SFAs) are those with no double bonds, and monounsaturated FAs (MUFAs) have 1 double bond. Polyunsaturated FAs (PUFAs) typically have 2 to 6 double bonds, and these are usually separated by a methylene group (CH_2 ; Budge et al. 2006). FAs are named using the notation A:Bn-x, where A is the number of carbons, B is the number of double bonds, and x is the position of the first double bond relative to the terminal methyl group. A list of abbreviations is given in Table 1.

FAs that are thought to convey dietary information are referred to in the literature as markers, indicators, and tracers, and these terms are used somewhat interchangeably. For the purpose of this review, we define a marker FA as one with an origin that can be unambiguously identified within the habitat of interest. Since markers unique to a given taxonomic group are relatively rare, we also discuss indicator FAs as those that are elevated in certain taxa, but may be found at lower concentrations in others. Both the presence and relative abundance of indicator FAs should therefore be taken into consideration when using these FAs to infer diet. We use 'tracer' as a general term that includes the use of marker or indicator FAs to track the flow of material through food webs.

Table 1. Abbreviations used throughout this review

FA	Fatty acid
SFA	Saturated FA: FA with no double bonds
MUFA	Monounsaturated FA: FA with 1 double bond
PUFA	Polyunsaturated FA: FA with ≥ 2 double bonds
HUFA	Highly unsaturated FA: PUFA with ≥ 4 double bonds
LCFA	Long-chain FA: FA with ≥ 24 carbons
C_x	Refers to the number of carbons in FA, e.g. C_{18} = 18 carbons
SI	Stable isotope

PRIMARY PRODUCER AND BACTERIAL FA

Phytoplankton are the base of pelagic food webs, and the main taxa of pelagic phytoplankton have distinct FA compositions (Dalsgaard et al. 2003). These microalgae also contribute to benthic food webs, and in some cases the presence of their characteristic FAs in benthic organisms indicates a trophic connection between pelagic and benthic food webs (Kharlamenko et al. 1995, Graeve et al. 1997, Parrish et al. 2009). Benthic food webs also rely on macroalgae, vascular plants (such as mangroves and seagrass), and bacteria, and these food sources also have characteristic FA compositions that can be used to identify their contributions to higher trophic levels (Dalsgaard et al. 2003).

To identify potential indicator FAs and compare overall FA composition of marine primary producers, we used similarity-based multivariate techniques (see 'Statistical analysis of FA data') to analyze FA data from 6 taxonomic groups of primary producers from 27 published studies (Table 2). While the focus of this review is on benthic systems, we have included phytoplankton FA data in this analysis because they enter benthic food webs as phytodetritus or by being consumed by suspension feeders. There were significant differences in overall FA composition among vascular plants, diatoms, dinoflagellates, and green, brown, and red macroalgae (analysis of similarity, ANOSIM, $R = 0.793$, $p < 0.001$). These taxa generally were separated in multivariate space using a 2-dimensional multidimensional scaling (MDS) plot (Fig. 1), although the stress value (0.21) is relatively high (stress = 0.13 for 3-dimensional MDS). All pairwise comparisons were significant ($p < 0.001$), although green algae and vascular plants were not well separated (ANOSIM, $R = 0.47$) compared to other groups ($R > 0.7$, see 'Statistical analysis of FA data'). We used similarity percentage analysis (SIMPER) to identify FAs that contributed at least 10% to within-group similarity for only 1 group: 18:1n-7 and 16:4n-3 for green algae; 18:4n-3 for brown algae; 22:6n-3 for dinoflagellates; and 16:1n-7 and 16:3n-4 for diatoms (Table 3). However, we also found several commonly-used indicators that were identified as contributing at least 10% to within-group similarity for multiple groups: 20:5n-3 was identified for dinoflagellates, diatoms, and brown and red macroalgae; 20:4n-6 for both brown and red algae; 18:1n-9 for brown and red algae, vascular plants, and dinoflagellates; and 18:2n-6 and 18:3n-3 for green algae and vascular plants (Table 3). This overlap indicates the need for caution when interpreting single FAs as

tracers for primary producers (Sargent et al. 1987, Dalsgaard et al. 2003).

Algae

De novo synthesis of algal FAs occurs in the chloroplasts and results in the production of SFAs, typically 16:0, from acetyl-CoA (Gurr et al. 2002). These SFAs are modified through a series of elongation and desaturation steps to produce various unsaturated FAs (Fig. 2). Conversion from 18:1n-9 to 18:2n-6 and 18:3n-3 requires $\Delta 12$ and $\Delta 15$ desaturase enzymes found only in primary producers. These 2 PUFAs are therefore called 'essential' FAs because animals must obtain them from their diets rather than by modifying other FAs (Dalsgaard et al. 2003). Further elongation and desaturation of 18:2n-6 and 18:3n-3 produce other common marine PUFAs including 20:4n-6, 20:5n-3, and 22:6n-3 (Gurr et al. 2002; Fig. 2). An alternative pathway, in which 16:0 is desaturated to 16:1n-7 and then further desaturated to 16:4n-1, is characteristic of diatoms, and both of these FAs are commonly used as indicators for diatoms (Dalsgaard et al. 2003).

The contributions of major microalgal taxa to benthic food webs are often estimated based on FA composition (Graeve et al. 2001, Howell et al. 2003, Guest et al. 2008). Diatoms and dinoflagellates have characteristic FA compositions that have been used as indicators in environmental samples (e.g. sediment or suspended matter) and in consumer tissues (Dalsgaard et al. 2003). Diatoms are frequently found in benthic environments as epiphytes (Nichols et al. 1986, Latyshev et al. 2004, Richoux & Froneman 2008), and both diatoms and dinoflagellates are captured from the water column by suspension feeders and from sediments by deposit feeders (Graeve et al. 2001, Howell et al. 2003, Guest et al. 2008). Diatoms are characterized by relatively high concentrations of 16:1n-7, 16:4n-1, and 20:5n-3, while dinoflagellates are typically rich in 22:6n-3 and 18:4n-3 (Budge & Parrish 1998, Dalsgaard et al. 2003). FA ratios are often used to distinguish between diatom and dinoflagellate dominance in phytoplankton samples: $16:1n-7/16:0 > 1$ or $20:5n-3/22:6n-3 > 1$ indicate diatom dominance (Budge & Parrish 1998, Dalsgaard et al. 2003; our Appendix 1). In our analysis of primary producer FA composition, diatoms and dinoflagellates are distinguished from each other by 16:1n-7 and 20:5n-3, which are more abundant in diatoms, and by 22:6n-3 and 18:1n-9, which are more abundant in dinoflagellates (Table 3).

Table 2. Studies used for analysis of primary producer fatty acid composition data (total 210 records, 111 species)

Taxon	No. of species	Location	Source
Phylum Ochrophyta,	14	Cultured	Dunstan et al. (1994)
Class Bacillariophyceae	6	Darwin Harbour, Australia	Renaud et al. (1994)
(diatoms)	2	Gulf of Carpentaria, Australia	Renaud et al. (1999)
	2	Queensland, Aus	Renaud et al. (1999)
	1	SE South Africa	Richoux & Froneman (2008)
	6	Cultured	Viso & Marty (1993)
	4	Cultured	Volkman et al. (1989)
Phylum Dinoflagellata	5	Cultured	Mansour et al. (1999)
(dinoflagellates)	1	Penang, Straits of Malacca	Usup et al. (2008)
	3	Sebatu, Straits of Malacca	Usup et al. (2008)
	6	South China Sea	Usup et al. (2008)
	3	Cultured	Viso & Marty (1993)
Phylum Chlorophyta	2	Brittany Coast, France	Fleurence et al. (1994)
(green algae)	1	Antarctic	Graeve et al. (2002)
	1	Spitsbergen, Arctic	Graeve et al. (2002)
	1	Nova Scotia, Canada	Kelly et al. (2009)
	2	Indian Ocean	Khotimchenko (2003)
	1	Sea of Japan	Khotimchenko (2003)
	1	South China Sea	Khotimchenko (2003)
	1	Yellow Sea	Khotimchenko (2003)
	3	Northern California, USA	Khotimchenko et al. (2002)
	3	Bohai Sea	Li et al. (2002)
	1	Okinawa, Japan	Meziane & Tsuchiya (2002)
	1	Southern California, USA	Nelson et al. (2002)
	1	Northern Chile	Ortiz et al. (2009)
	1	SE South Africa	Richoux & Froneman (2008)
	1	Sea of Japan	Sanina et al. (2004)
	8	Yellow Sea	Vaskovsky et al. (1996)
Phylum Ochrophyta,	1	Northern New Zealand	Alfaro et al. (2006)
Class Phaeophyceae	5	Brittany Coast, France	Fleurence et al. (1994)
(brown algae)	3	Antarctica	Graeve et al. (2002)
	3	Spitsbergen, Arctic	Graeve et al. (2002)
	1	Cultured	Honya et al. (1994)
	3	Nova Scotia, Canada	Kelly et al. (2008)
	4	Kurile Islands	Khotimchenko (1998)
	15	Sea of Japan	Khotimchenko (1998)
	2	Sea of Japan	Khotimchenko & Kulikova (1999)
	9	Northern California, USA	Khotimchenko et al. (2002)
	10	Bohai Sea	Li et al. (2002)
	1	Southern California, USA	Nelson et al. (2002)
	1	Cultured	Ortiz et al. (2009)
	2	Sea of Japan	Sanina et al. (2004)
	6	Yellow Sea	Vaskovsky et al. (1996)
Phylum Rhodophyta	4	Brittany Coast, France	Fleurence et al. (1994)
(red algae)	11	Antarctica	Graeve et al. (2002)
	4	Spitsbergen, Arctic	Graeve et al. (2002)
	1	Nova Scotia, Canada	Kelly et al. (2008)
	1	Sea of Japan	Khotimchenko (2005)
	7	Northern California, USA	Khotimchenko et al. (2002)
	9	Bohai Sea	Li et al. (2002)
	1	Southern California, USA	Nelson et al. (2002)
	1	Cultured	Ortiz et al. (2009)
	12	Yellow Sea	Vaskovsky et al. (1996)
Phylum Tracheophyta	2	Northern New Zealand	Alfaro et al. (2006)
(vascular plants)	1	NE Australia	Hall et al. (2006)
	1	Sea of Japan	Kharlamenko et al. (2001)
	1	Okinawa, Japan	Meziane & Tsuchiya (2002)
	4	SE South Africa	Richoux & Froneman (2008)
	2	Yellow Sea	Vaskovsky et al. (1996)

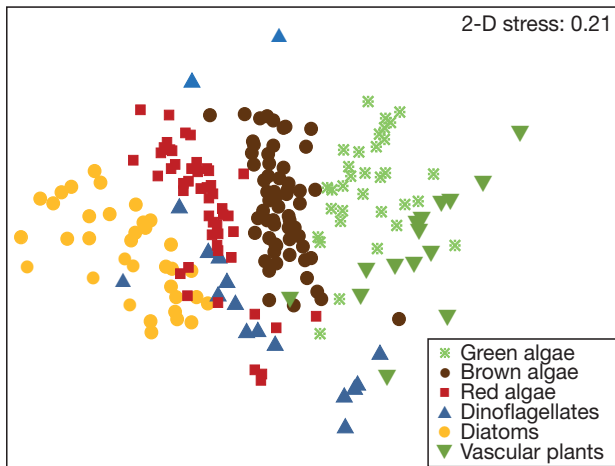


Fig. 1. Non-metric multidimensional scaling (MDS) plot based on Bray-Curtis similarities of fatty acid (FA) data for 6 taxa of primary producers (see Table 2). FAs with average contribution $\geq 0.1\%$ were included; data were standardized to equal 100% prior to analysis. Each point represents a species from a particular field site or laboratory culture

Table 3. Results of similarity percentage analysis (SIMPER) of fatty acid (FA) data for primary producers (see Table 2). FAs with average contribution $\geq 0.1\%$ included; data were not transformed prior to analysis

Taxon	FA	Average abundance	Contribution to similarity (%)
Phylum Chlorophyta (62% average similarity)	16:0	29.96	36
	18:3n-3	20.42	26
	18:2n-6	7.24	7
	18:1n-7	7.15	6
	16:4n-3	6.44	4
Class Phaeophyceae (70% average similarity)	16:0	21.85	25
	18:1n-9	14.15	14
	20:4n-6	10.53	11
	20:5n-3	11.73	11
	18:4n-3	10.89	10
Phylum Rhodophyta (66% average similarity)	16:0	34.66	44
	20:5n-3	30.34	31
	18:1n-9	7.37	7
	20:4n-6	8.40	5
	14:0	4.45	5
Phylum Dinoflagellata (48% average similarity)	16:0	26.92	42
	18:1n-9	13.70	15
	22:6n-3	13.14	12
	20:5n-3	10.39	10
	18:0	7.49	7
Class Bacillariophyceae (65% average similarity)	16:1n-7	25.97	33
	16:0	19.42	21
	20:5n-3	15.19	15
	14:0	13.51	14
	16:3n-4	6.14	4
Phylum Tracheophyta (56% average similarity)	16:0	26.08	38
	18:3n-3	30.32	34
	18:2n-6	13.88	12
	18:1n-9	4.50	4
	18:0	3.48	4

Macroalgae are rich in PUFAs, and contain especially high concentrations of n-6 PUFAs as compared with microalgae (Dalsgaard et al. 2003). Red algae typically contain a high concentration of 20:5n-3 (as high as 67% in *Adouinella purpurea*, Graeve et al. 2002) and moderate levels of 20:4n-6, 18:1n-9, and 16:1n-7 (Vaskovsky et al. 1996, Graeve et al. 2002, Li et al. 2002). Rhodophyta is the oldest macroalgal lineage, and dominance of C₂₀ PUFAs is considered an ancestral characteristic (Graeve et al. 2002). Green algae contain a higher concentration of C₁₈ PUFAs (18:3n-3, 18:2n-6, 18:4n-3) and lower levels of C₂₀ PUFAs than other macroalgae (Vaskovsky et al. 1996, Khotimchenko et al. 2002, Li et al. 2002). Certain green algae also contain high levels of 16:4n-3 (10 to 20% in the order Ulvales; Fleurence et al. 1994, Vaskovsky et al. 1996, Khotimchenko et al. 2002). Green algae represent the most modern macroalgal lineage, and their FA composition is most similar to that of higher plants,

which are generally dominated by C₁₈ PUFAs (Graeve et al. 2002). In our analysis, green and red macroalgae are distinguished mainly by 20:5n-3, which is more abundant in red algae, and 18:3n-3, which is more abundant in green algae (Table 3). Brown algae are characterized by relatively high concentrations of 20:5n-3, 20:4n-6, 18:1n-9, and 18:4n-3, with slightly lower concentrations of 18:2n-6 and 18:3n-3 (Vaskovsky et al. 1996, Graeve et al. 2002, Khotimchenko et al. 2002, Li et al. 2002). This mixed FA composition supports the intermediate phylogenetic position of the Phaeophyta (brown algae) between the older Rhodophyta (red algae) and more modern Chlorophyta (green algae; Graeve et al. 2002). SIMPER analysis showed that the FAs that contribute most to differences between brown and green macroalgae are 18:3n-3 (more abundant in green algae) and 18:1n-9 (more abundant in brown algae). Brown and red macroalgae are distinguished by 20:5n-3 and 16:0, which are more abundant in red algae, and by 18:4n-3, 20:4n-6, and 18:1n-9, which are more abundant in brown algae (Table 3). Considerable seasonal variability in FA composition has been found in macroalgae (Honya et al. 1994, Nelson et al. 2002), and sampling for FA characterization ideally should span all seasons to capture this variation.

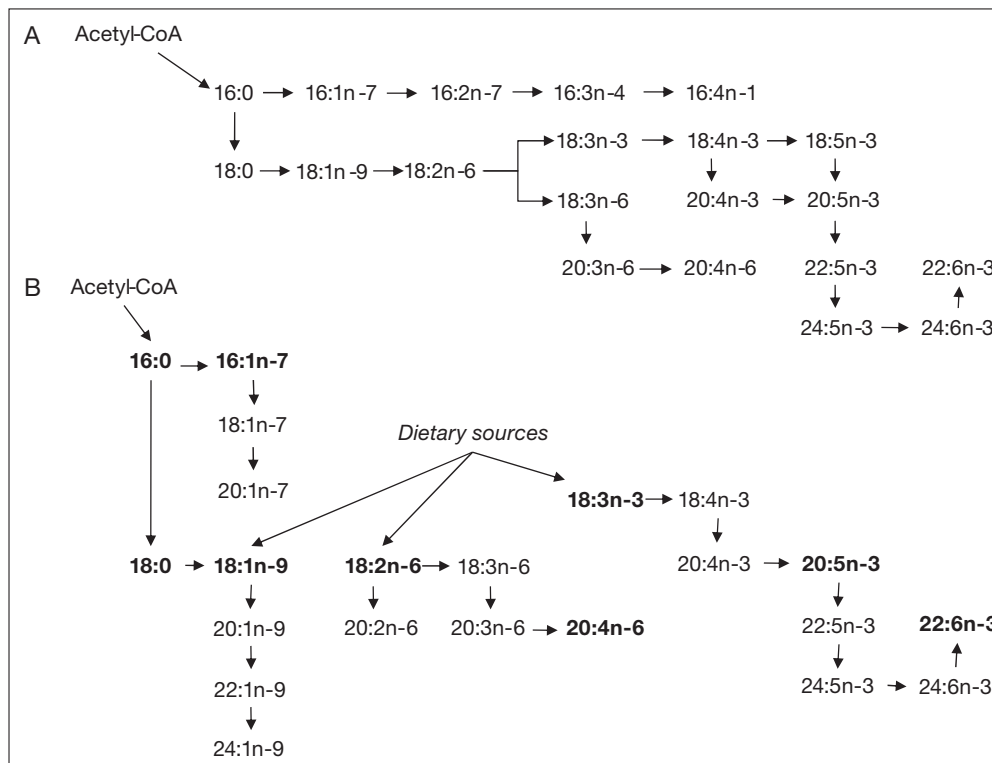


Fig. 2. Major fatty acid (FA) biosynthetic pathways in (A) plants and algae and (B) animals. FAs shown in **bold** in (B) tend to accumulate at relatively high concentrations in animal tissues. Horizontal arrows represent desaturation, downward vertical arrows represent 2-carbon chain elongation, and upward vertical arrows represent β -oxidation. Modified after Gurr et al. (2002) and Bergé & Barnathan (2005)

Vascular plants

Vascular plants contribute to nearshore benthic food webs through direct consumption by grazers and via detrital pathways (Meziane et al. 1997, Hall et al. 2006). As in green algae, PUFAs in vascular plants consist mainly of 18:2n-6 and 18:3n-3 (Dalsgaard et al. 2003). Saturated, long-chain FAs (LCFAs) with an even number of carbon atoms ($\geq C_{22}$) are found in the waxy leaf cuticle of higher plants; along with 18:2n-6 and 18:3n-3, these LCFAs serve as indicators of vascular plant matter in sediments and suspended organic matter (Canuel et al. 1995, Budge et al. 2001). LCFAs have also been used as trophic markers for mangroves and halophytes in benthic invertebrates (Meziane et al. 1997, Meziane & Tsuchiya 2000, 2002), but they are not assimilated by all consumers and may be of limited use as tracers in some food webs (Hall et al. 2006). SIMPER analysis showed that vascular plants differ from green algae in having higher relative abundance of 18:3n-3, 18:2n-6, and LCFAs (Table 3).

Marine bacteria

Both autotrophic and heterotrophic bacteria play an important role in many benthic food webs, as food sources for deposit and suspension feeders, and as endosymbionts of invertebrates (Kharlamenko et al. 1995, Pond et al. 1997, 2002, Meziane & Tsuchiya 2002, Howell et al. 2003, McLeod & Wing 2009). FA composition of cyanobacteria is generally dominated by 16:0 and 14:0, with lower concentrations of 18:0, 16:1n-9, and 18:1n-9 (Carpenter et al. 1997). FA biosynthesis in heterotrophic bacteria produces odd-numbered and branched FAs such as 15:0, 17:0, 15:1, 17:1, and *iso*- and *anteiso*-branched SFAs (Dalsgaard et al. 2003). These FAs, along with 16:1n-7 and 18:1n-7, are typically dominant in bacterial FA composition and are used as tracers for the contribution of heterotrophic bacteria to sediments, suspended organic material, and animal diets (Kharlamenko et al. 1995, Meziane & Tsuchiya 2002, Dalsgaard et al. 2003). Production of PUFAs is relatively rare among bacteria, although PUFA synthesis has been shown in cold-water and deep-sea bacteria (Nichols et al.

1993, Russell & Nichols 1999). Relative proportions of various bacterial FAs are indicative of different taxonomic groups of bacteria (Sargent et al. 1987, Colaço et al. 2007; our Appendix 1), and have been used to examine microbial community structure in marine sediments (Parkes & Taylor 1983, Gillan & Hogg 1984, McLeod & Wing 2009).

CONTROLLED FEEDING STUDIES

Controlled feeding studies in a laboratory setting can further our understanding of how FAs are transferred from food to consumer, which FAs are catabolized or excreted, and how specific FAs affect consumer growth and physiology. Field studies in which diet is inferred from FAs present in consumers generally assume that FAs are transferred from food to consumer with minimal modification. This is a reasonable assumption in some taxa; for example, mammalian predators have a very limited capacity to alter dietary FAs (Budge et al. 2006). However, because benthic invertebrates have a greater ability to modify dietary FAs (Dalsgaard et al. 2003, Bell & Tocher 2009), controlled feeding studies should be employed before using FAs as dietary tracers in field studies. Many controlled feeding studies of benthic organisms have been undertaken for aquaculture-related purposes, such as maximizing growth, reproduction, or larval survival (e.g. Glencross & Smith 1999, Castell et al. 2004, Calado et al. 2005). Diets in such studies often include feeds with high concentrations of specific FAs, making it possible to isolate the effects of FAs of interest (Castell et al. 2004, Calado et al. 2005). However, the diets used in such studies typically bear little resemblance to natural diets, and as such the results generally are not useful for determining the diets of animals in the wild. More controlled feeding studies using natural diets are required for validation of indicator FAs and to improve understanding of biosynthesis and selective retention. These studies can also help to quantify variation in FAs among tissue types, life history stages, and reproductive phases, as these factors can have a significant impact on FA composition (Hughes et al. 2006, Mateos et al. 2010).

FA transfer

Controlled feeding studies have identified FAs that transfer reliably from food to consumer. These FAs may be useful as indicators for identifying certain food sources in the diets of consumers if their concen-

trations are found to be (1) significantly higher in 1 food source than others and (2) significantly higher in consumers of the food source of interest as compared to consumers of other foods. Most studies that have demonstrated FA transfer in benthic invertebrates have used artificial diets enriched with high concentrations of certain FAs (Castell et al. 2004, Suprayudi et al. 2004, Liu et al. 2007).

The results of comparisons among natural diets are likely more applicable to ecologists seeking tracers for use in field studies than are aquaculture-focused studies comparing artificial diets, but controlled feeding studies comparing natural foods are relatively few. Indicator FAs for rotifers, diatoms, dinoflagellates, kelps, and green algae have been identified in their various invertebrate consumers (D'Souza & Loneragan 1999, Shin et al. 2008, Kelly et al. 2009). However, when sea urchins *Strongylocentrotus droebachiensis* were fed 4 different algal diets, no indicators distinguished sea urchins among diet treatments (Kelly et al. 2008).

Tracking changes in FA composition over time during a controlled feeding experiment may also be useful for validating FA indicators for a diet of interest. When sea urchins *Strongylocentrotus droebachiensis* were switched from kelp to a grain-based diet, kelp indicators decreased, and indicators for the artificial diet increased in sea urchin tissues (Liyana-Pathirana et al. 2002). Both mangrove leaves and the tissues of crabs *Parasesarma erythodactyla* fed these leaves contained high levels of 18:2n-6 and 18:3n-3 (Hall et al. 2006). A feeding/starving regimen provided further support for these FAs as dietary indicators: levels of both FAs increased when crabs were fed mangrove leaves, and decreased during starvation periods. 18:3n-3 had similar indicator properties when tissues of *P. erythodactyla* were fed to another crab (*Portunus pelagicus*). However, both FAs are common in primary producers and may be of limited use in field studies (Hall et al. 2006).

FA biosynthesis and selective retention

Many invertebrate taxa possess active elongase and desaturase enzymes that enable them to significantly modify dietary FAs (Spychalla et al. 1997, D'Souza & Loneragan 1999, Bell et al. 2001, Hall et al. 2006). Crabs *Parasesarma erythodactyla* and *Portunus pelagicus* and larval prawns *Penaeus* spp. synthesized highly unsaturated FAs (HUFAs) when these were absent from their diets, suggesting biosynthesis from dietary precursors (D'Souza & Loneragan 1999).

gan 1999, Hall et al. 2006). Bell et al. (2001) used a radio-labeled diet to show that the sea urchin *Psammechinus miliaris* can convert 18:3n-3 to 20:5n-3, even when 20:5n-3 is present in the diet, but that the rate of conversion is very slow. Because marine fish and mammals cannot convert 18:2n-6 and 18:3n-3 to the HUFAs 20:4n-6, 20:5n-3, and 22:6n-3, and must obtain these FAs from their diets, biosynthesis by invertebrates may represent an important link in marine food webs (Sargent et al. 1999, Hall et al. 2006).

Products of biosynthesis may be useful as dietary indicators if there is a clear relationship between levels of the precursor FAs in the food and the product in the consumer. Sea urchins *Strongylocentrotus droebachiensis* fed artificial diets rich in 18:1n-9 had high concentrations of both 18:1n-9 and its elongation product 20:1n-9 (Castell et al. 2004). Levels of HUFAs in sea urchins were directly related to levels of C₁₈ precursor FAs in HUFA-deficient artificial diets (Castell et al. 2004). The use of products of FA biosynthesis as dietary indicators merits further research, as these may prove useful in field studies where few dietary indicator FAs are transferred directly to consumer tissues.

Either biosynthesis or selective retention may be postulated when consumer tissues contain higher concentrations of certain FAs than does their food (Castell et al. 2004). Controlled feeding studies of larval and adult sea urchins have found relatively high concentrations of HUFAs and C₂₀ and C₂₂ MUFAs in sea urchin tissues (5 to 18%) when these were present only in trace amounts (<0.1%) in their diets (Castell et al. 2004, Liu et al. 2007, Kelly et al. 2008). The magnitude of the increase between diet and tissues, in addition to the presence of C₁₈ precursors in the diets, suggests biosynthesis rather than selective retention by sea urchins. In contrast, juvenile shrimp *Lysmata seticaudata* contained higher HUFA concentrations than did their food, but the very low concentrations of C₁₈ precursor FAs in their food suggested selective retention rather than biosynthesis (Calado et al. 2005). Both biosynthesis and selective retention of HUFAs are likely to occur only when the diet is deficient in these (Iverson 2009). For example, Cook et al. (2000) found that sea urchins *Psammechinus miliaris* selectively retained HUFAs 20:4n-6 and 20:5n-3 when fed salmon feed pellets that were deficient in these, but not when fed kelp, their natural diet, which contained higher concentrations of both HUFAs. FA data from controlled feeding experiments should be carefully examined for evidence of biosynthesis and selective retention to distinguish FAs affected by these processes from those that are directly deposited from diet.

Egested FAs

Consumers may egest certain FAs rather than assimilating them directly or after modification. Mangrove leaves contained 17 to 24% LCFAs (24:0, 26:0, and 28:0), but tissues of crabs *Parasesarma erythro-dactyla* fed mangrove leaves did not contain these FAs (Hall et al. 2006). However, feces of *P. erythro-dactyla* contained over 15% LCFAs, suggesting that the crabs may be unable to metabolize these FAs (Hall et al. 2006). These results highlight the need for controlled feeding studies before FAs are used as tracers in field studies: the lack of LCFAs in tissues of *P. erythro-dactyla* could otherwise appear to indicate that these crabs do not feed on mangrove leaves. Fecal pellets of deposit-feeding fiddler crabs *Uca vocans* and *Scopimera globosa* also contained LCFAs that were absent from their tissues (Meziane et al. 2002). These feces also contained lower levels of indicator FAs for bacteria and diatoms than did tissues and sediments, suggesting that the crabs effectively assimilated diatoms and bacteria but not mangrove leaves (Meziane et al. 2002). Comparisons of FA profiles of consumer feces to those of food and consumer tissues may provide valuable information in other benthic systems and should be included in controlled feeding studies.

FA ANALYSIS OF BENTHIC FOOD WEBS

FA analysis has been used in field studies to address a variety of questions related to trophic structure in benthic habitats. One relatively common use is to infer diet from consumer FAs. However, predicting consumer diets based on a few FAs can be problematic, since most marine FAs are ubiquitous and therefore rarely indicate the presence of a specific food item. Use of a single or few FAs can also be misleading in that these FAs could be obtained by consuming a primary producer, or by preying on a consumer of that primary producer (Budge et al. 2006). FAs are frequently used as tracers in field studies without considering the role of consumer metabolism, even though this may play a significant role in determining consumer FA composition. Field studies that use tracers validated in controlled feeding studies are rare (Cook et al. 2000), but such studies are important because they allow comparison between consumers fed a single diet and wild-caught animals that likely consumed a mixed diet, and provide some indication of which FAs are affected by biosynthesis or selective retention. Ideally, field stud-

ies should employ multivariate analyses to examine overall patterns and to identify FAs that are important in separating groups in addition to univariate comparisons.

FAs are useful in habitats where direct observation would pose significant challenges, such as the deep sea (Howell et al. 2003, Drazen et al. 2009). FA tracers are also used to determine the fate of primary production, especially in habitats where the dominant primary producer is not directly consumed by the dominant herbivores (Meziane & Tsuchiya 2000, Kharlamenko et al. 2001). Other field studies have employed FA analysis to address ecological questions about how animal diets vary with habitat, season, and anthropogenic effects (Takagi et al. 1980, Hughes et al. 2005, Fernandez-Jover et al. 2007).

Deep sea and hydrothermal vent food webs

Deep sea food webs are especially well-suited to study using FA analysis because direct observation is difficult at depth, and food webs are relatively simple because of the limited number of primary producers. Studying the diet of deep sea species using more direct methods is problematic because the pressure changes experienced as animals are brought to the surface frequently causes regurgitation or stomach eversion, precluding analysis of stomach contents (e.g. sea stars, Howell et al. 2003; macrourid fishes, Drazen et al. 2009). Relative abundances of several diet-derived FAs varied among 7 species of deep sea holothurians from the northeast Atlantic, suggesting a diversity of feeding modes or niches (Ginger et al. 2000). Deep sea holothurians and ophiuroids from the northeast Pacific were distinguished by the higher proportions of algal-derived HUFAs in the deposit-feeding holothurians and by copepod-derived MUFAs in the omnivorous ophiuroids (Drazen et al. 2008a). When 8 species of deep-sea sea stars from the northeast Atlantic were classified according to their overall FA composition, their categories corresponded with their known feeding types: suspension feeders contained higher proportions of indicator FAs of diatoms, dinoflagellates, and copepods; and mud-ingesting sea stars contained higher proportions of bacterial FAs (Howell et al. 2003). Each of the 3 predator-scavenger species had a distinct FA composition, indicating specialization on (1) benthic invertebrates and foraminifera, (2) microinfauna, and (3) echinoderms (Howell et al. 2003). These studies demonstrate the utility of FA analysis in classifying benthic consumers (particularly those

that are difficult to sample and observe *in situ*) into various trophic groups.

Chemoautotrophic bacteria provide the major food source for invertebrate communities at deep sea hydrothermal vents, and their characteristic FA profile allows these bacteria to be traced through the food web. In deep sea habitats, 16:1n-7 and 18:1n-7 indicate bacterial food sources, especially when 20:1n-7 and 22:1n-7 are present and the microalgal indicator FAs 20:5n-3 and 22:6n-3 are absent (Ben-Mlih et al. 1992). These bacterial indicator FAs have been found in several hydrothermal vent invertebrates including deep sea mussels *Bathymodiolus thermophilus* (Ben-Mlih et al. 1992, Phleger et al. 2005), shrimp *Rimicaris exoculata* (Pond et al. 1997), clams *Calyptogena pacifica* (Allen et al. 2001), and tubeworms *Riftia pachyptila* (Phleger et al. 2005). Colaço et al. (2007) classified invertebrates from deep hydrothermal vents at the Mid-Atlantic Ridge into 2 groups based on their overall FA composition: (1) mussels, their commensal polychaetes, and whelks, which all contained a higher proportion of branched n-7 FAs, indicating a reliance on sulfide-reducing bacteria; and (2) shrimp and crabs, which contained a higher proportion of n-7 MUFA and non-methylene-interrupted (NMI) FAs, indicating a reliance on sulfate-oxidizing bacteria. The deposit-feeding polychaete *Amathys lutzi* contained indicator FAs of both types of bacteria, indicating that it feeds non-selectively (Colaço et al. 2007). In addition to bacterial FAs, shrimp *Alvinocaris markensis* from the Juan de Fuca Ridge contained high proportions of HUFAs 20:5n-3 and 22:6n-3. These HUFAs are often considered markers of phytodetritus (i.e. diatoms and dinoflagellates), but 22:6n-3 in particular may also indicate a scavenging diet, and appendages of *A. markensis* are adapted for scavenging (Pond et al. 1997). Low proportions of 20:5n-3 and 22:6n-3 in hydrothermal vent mussels *B. thermophilus* (Ben-Mlih et al. 1992) and clams *C. pacifica* (Allen et al. 2001) are likely to be photosynthetically-derived and acquired by filter feeding. Tubeworms *Ridgeia piscesae* and *Protis hydrothermica* from hydrothermal vents were rich in bacterial indicator FAs, but also in 20:5n-3 and 20:4n-6 (Pond et al. 2002). *R. piscesae*, which relies entirely on bacterial endosymbionts for nutrition, has the enzymes required to synthesize these HUFAs from bacterially derived C₁₈ precursors (Pond et al. 2002). This underscores the importance of understanding the biosynthetic capabilities of animals before attempting to infer diet from FA composition.

Like their deep-water counterparts, shallow hydrothermal vent systems are host to chemoautotrophic

bacteria (Kharlamenko et al. 1995). However, animals in shallow vent systems have access to a wider variety of potential food sources, including planktonic diatoms, particulate organic matter, microbial mats, sediments, and macrophytes, and may therefore have more complex trophic pathways. Based on their overall FA profiles, 16 consumer taxa at a shallow hydrothermal vent off the Kurile Islands in the northwest Pacific were classified into 7 clusters that corresponded generally with their feeding modes. Suspension feeders had high levels of the diatom indicator 20:5n-3, while carnivorous polychaetes and corals clustered together in multivariate space and had high levels of 22:6n-3. Sea cucumbers *Eupentacta pseudoquinquesemita* and *Psolus* spp. had high bacterial FA concentrations, which point to deposit feeding, and also relatively high concentrations of 20:5n-3, suggesting diatoms as a likely food source. These species are suspension feeders, so the high concentration of 20:5n-3 was expected, and dissection of gill tissues revealed symbiotic bacteria (rather than the microbial mat) as the source of bacterial FAs (Kharlamenko et al. 1995).

Polar food webs

FA analysis of benthic fauna in polar habitats has improved our understanding of their trophic interactions. In the Arctic, suspension-feeding brittle stars were rich in indicator FAs for diatoms (Graeve et al. 1997). Benthic decapod crustaceans in the Arctic contained high concentrations of both diatom and carnivory indicators, suggesting that their prey consumed diatoms (Graeve et al. 1997). FA profiles of 6 species of Antarctic amphipods corresponded with their feeding modes: for example, the grazer *Oradarea edentata* was rich in the macroalgal indicator 20:4n-6 (Graeve et al. 2001).

Indicator FAs have also been used to identify diets of higher consumers in polar food webs. In the Antarctic, FA compositions of benthic icefishes *Dolloidraco longedorsalis* and *Bathydraco marri* were similar to that of their benthic invertebrate prey in that all were rich in diatom and carnivory indicator FAs (Hagen et al. 2000). Copepod indicators (20:1 and 22:1 MUFAs) comprised <5% of total FAs in benthic icefishes, as compared with 10 to 15% in pelagic species (Hagen et al. 2000). In the Arctic, FA composition of Pacific walrus *Odobenus rosmarus* was distinct from that of bearded seals *Erignathus barbatus* based on the higher ratio of n-7 to n-9 isomers of C₁₈–C₂₂ MUFAs in walrus (Budge et al. 2007).

A high n-7 to n-9 ratio is characteristic of benthic mollusks, which are thought to be the major prey of walrus. The 2 species also differed in the composition of their NMI FAs, suggesting that levels of individual NMI FAs may be useful as species-specific markers for certain benthic invertebrates in the diets of higher consumers (Budge et al. 2007).

Estuarine food webs

Estuarine habitats support relatively diverse faunal assemblages, but few of the resident species directly consume the dominant macrophytes, such as mangroves and seagrasses (Meziane & Tsuchiya 2000, Kharlamenko et al. 2001). FA analysis has been used to trace the fate of these primary producers and to compare the relative contribution of mangrove and seagrass material to consumer diets with that of other food sources such as macroalgae, diatoms, dinoflagellates, zooplankton, and bacteria. The diversity of potential food sources in these habitats requires that their FAs be characterized before attempting to infer consumer diets, and the overlap in indicator FAs among common food sources (Appendix 1) points to the need for multivariate analysis rather than interpretation of single FAs. Many of the field studies using FAs to identify diets of estuarine consumers have relied entirely on comparisons between consumers and their potential food sources (e.g. Kharlamenko et al. 2001, Alfaro et al. 2006), without controlled feeding studies that demonstrate trophic transfer of certain indicator FAs.

Based on indicator FAs present in consumer tissues, Kharlamenko et al. (2001) concluded that most animals in a seagrass bed in the Sea of Japan consumed seagrass as detritus or suspended particulate matter, and that diet of deposit feeders was more varied than that of grazers. Several mollusks in the study contained relatively high levels of 20:4n-6, which was attributed to consumption of fungi, even though fungi were not analyzed and 20:4n-6 was found only in very low levels in sediments and suspended particulate matter. Biosynthesis of 20:4n-6 from its precursor 18:2n-6 may have been a more likely source of this FA, and has been demonstrated in controlled feeding studies of the scallop *Pecten maximus* (Soudant et al. 1996).

Most consumers in a tropical mangrove- and seagrass-dominated estuary in northern New Zealand utilize a variety of food sources including mangrove leaves, seagrass, microalgae, a brown macroalga, zooplankton, and bacteria (Alfaro et al. 2006). Alfaro et al. (2006) interpreted the presence of 18:3n-3 con-

servatively, since it was present in both seagrass and mangrove leaves. This relatively cautious approach to interpreting FA indicator data is particularly appropriate when controlled feeding data are not available for consumers, and therefore their capacity for FA modification is unknown. Data from Alfaro et al. (2006) show evidence of biosynthesis or selective retention of PUFAs by some consumers. For example, 20:5n-3 accounted for 17.7% of FAs in the shrimp *Palaemon affinis*, while the highest concentration of this FA in any potential food source was 6.0% in sediment. Many invertebrates are capable of synthesizing 20:5n-3 using 18:3n-3 as a precursor (e.g. crab *Parasquilla erythodactyla*, Hall et al. 2006; sea urchin *Strongylocentrotus droebachiensis*, Castell et al. 2004). Relative abundance of 18:3n-3 in primary producers ranged from 3.0% in the brown macroalgae *Hormosira banksii* to 27.4% in seagrass, but its maximum abundance in consumers was 2.1%. This suggests that 18:3n-3 was usually catabolized by consumers and was probably not useful as an indicator, although this was not considered by Alfaro et al. (2006). This underscores the need for controlled feeding studies to distinguish FAs that are reliable indicators from those that are affected by consumer metabolism.

Food webs of temperate estuaries are similar to those in the tropics in that most consumers are trophic generalists, and most of the biomass of dominant primary producers enters the detrital food web rather than being directly grazed (Meziane et al. 1997, Richoux & Froneman 2008). Although 10 food sources in a temperate South African estuary had distinct FA compositions, relatively few indicators for these food sources were useful in distinguishing among 14 consumer species (Richoux & Froneman 2008). Multivariate analyses of FA composition separated consumers into 2 general groups based on dominance of either dinoflagellates or benthic diatoms in their diets (Richoux & Froneman 2008). Benthic diatoms appear to play an important role in temperate estuarine food webs: the diatom indicators 16:1n-7 and 20:5n-3 comprise significant proportions of total FAs in sediments and animals, but not in suspended material (Meziane et al. 1997, Richoux & Froneman 2008).

Rocky subtidal food webs

Relatively few studies have used FAs to analyze rocky subtidal food webs, and a majority of these studies have focused on identifying diets of sea urchins. FA composition of sea urchins *Strongylocen-*

trotus droebachiensis from a kelp bed off the Atlantic coast of Nova Scotia, Canada, differed from that of sea urchins from nearby coralline algae-dominated barrens (Takagi et al. 1980). Most notably, both kelp and sea urchins from the kelp bed were rich in 20:4n-6, while sea urchins from barrens contained a higher proportion of n-3 PUFAs. Intertidal and subtidal populations of the sea urchin *Psammechinus miliaris* off the west coast of Scotland differed in their FA composition, but these differences were not consistent between sites, suggesting that sea urchin diets are variable (Hughes et al. 2005). FA composition was not reported for the dominant macroalgae, but dominant taxa in the intertidal (furoid algae) and subtidal (laminarian kelp) zones may not have been sufficiently distinct in their FA composition to allow differentiation at the next trophic level. Intertidal sea urchins at 1 site contained a higher concentration of the carnivory indicator 22:6n-3, suggesting that invertebrate prey contributed to their diet (Hughes et al. 2005). As in estuarine food webs, the utility of FAs as dietary tracers in the rocky subtidal zone is limited because of the diversity of potential food sources and lack of unique markers. In both systems, it appears that FAs should be used primarily for classifying consumers into broad dietary groups unless controlled feeding studies have been used to verify the trophic transfer of specific FAs.

Aquaculture effects on food webs

The effects of fish farms on animal diets and trophic interactions are well-suited to FA analyses because fish feed tends to contain indicator FAs that distinguish it from other benthic food sources. Sea urchins *Psammechinus miliaris* living under a salmon pen off the west coast of Scotland contained high concentrations of 22:6n-3, which is a major constituent FA of the salmon feed (Cook et al. 2000). Sea urchins fed salmon feed pellets in the laboratory also contained high levels of 22:6n-3, confirming that 22:6n-3 is a valid indicator for this salmon feed in sea urchins. FAs 18:4n-3 and 20:4n-6 were similarly validated as indicators for kelp and were elevated in sea urchins from kelp-dominated intertidal sites (Cook et al. 2000). Invasive amphipods *Caprella mutica* collected from salmon pens, mussel longlines, and an artificial reef contained relatively high concentrations of 20:5n-3, 22:6n-3, and 18:1n-9, indicating an omnivorous diet including animal material and diatoms (Cook et al. 2010). Wild Mediterranean horse mackerel *Trachurus mediterraneus* that aggregated near fish pens off the south-east coast of Spain had

similar FA composition to feed pellets, in contrast to control populations distant from fish farms (Fernandez-Jover et al. 2007). The use of indicator FAs to detect non-native food sources in consumer diets is a promising application of FAs in benthic systems.

STATISTICAL ANALYSIS OF FA DATA

The large size of most FA datasets requires that overall patterns among samples be examined using a multivariate statistical approach. Similarity-based techniques may be most appropriate for FA data because they have fewer assumptions regarding data structure and can be used with small sample sizes. Univariate statistical methods can be used in conjunction with multivariate analyses to compare levels of individual tracer FAs among consumers and/or food sources, but care should be taken to minimize Type I errors associated with multiple comparisons. While a wide variety of statistical techniques could potentially be applied to FA data, we briefly review those that are most commonly used.

Multivariate statistics

Multivariate analysis of variance (MANOVA) and discriminant function analysis (DFA) are used to examine differences in overall FA composition among *a priori* groups of samples, e.g. different species (Budge et al. 2002), functional groups (Piché et al. 2010), or consumers on different diets in a controlled feeding study (Navarro et al. 1997). MANOVA is used to test for significant differences in overall FA composition among groups of samples by examining whether mean differences among groups could have occurred by chance. DFA calculates the combination of FAs that produces maximum multivariate distance among groups by creating uncorrelated linear equations of the original FAs. Scores of samples on the resulting discriminant functions can be plotted to reveal relationships among samples. Interpretation of DFA results is relatively simple, and FAs that are most important in separating groups along each function can usually be identified (Navarro et al. 1997, Budge et al. 2006). DFA can also be used to classify new samples into trophic groups based on FA composition (Hair et al. 2006).

Principal component analysis (PCA) is used as an exploratory technique to examine relationships among samples, and to simplify large FA datasets. PCA reduces large numbers of variables to a few

components that represent most of the variance in the data by combining correlated FA into new components. This allows identification of FAs that contribute most to separation among observed groups and FAs that are highly correlated (Budge et al. 2006). Tracer FAs for food sources of interest may be useful for interpreting PCA results where FAs that contribute to discriminating groups are also tracers. The scores of each sample on the first 2 to 3 principal components can be plotted to visualize relationships among samples. PCA has been used in benthic habitats to compare FA signatures of consumers to their potential food items (Guest et al. 2008, Drazen et al. 2009) and to classify consumers by feeding mode (Colaço et al. 2007, Drazen et al. 2008a, Richoux & Froneman 2008).

MANOVA and DFA assume that the data are multivariate normal and the covariance matrices are homogeneous (Hair et al. 2006). The assumptions of PCA are somewhat less stringent, although multivariate normality is preferred and more samples than variables are required for reliable results (Hair et al. 2006). Proportional FA data require transformation to meet the assumption of multivariate normality (Budge et al. 2006). The log-ratio transformation, $x_{\text{trans}} = \ln(x_i/c_r)$, where x_i is a given FA expressed as percent of total FAs, and c_r is the percentage of a reference FA, has been recommended for FA data (Budge et al. 2006, Piché et al. 2010). The reference FA for this transformation should be one that is present regardless of diet, and is reliably quantified (Budge et al. 2006). The arcsine transformation is also commonly used for proportional data and may improve normality in some cases (Cook et al. 2000, Castell et al. 2004, Thurber 2007). The logit transformation may be more suitable alternative (Warton & Hui 2011), but multivariate normality is difficult to achieve with large FA datasets, even after transformation (Budge et al. 2006). If normality is tested at all, it is usually for individual FAs (e.g. Shapiro-Wilk test), and not for the multivariate dataset (e.g. Henze-Zirkler test). Additionally, to avoid heterogeneity of covariance, the number of variables in FA datasets usually must be reduced to $n - 1$ where n is the smallest number of samples in any group, and each group should contain at least 20 samples (Budge et al. 2006, Hair et al. 2006). The subset of FAs retained for analysis should include those that are generally obtained from diet rather than from biosynthesis by the consumer, and those that are tracers of potential food items (Iverson et al. 2004, Budge et al. 2006). However, the selection of FAs that are of dietary rather than biosynthetic origin is often difficult for

benthic invertebrates because many species are capable of substantial FA biosynthesis (D'Souza & Loneragan 1999, Cook et al. 2000, Castell et al. 2004, Calado et al. 2005, Hall et al. 2006), and FA metabolism for most species is not well-studied. The cost of obtaining an adequate number of samples for these techniques may be prohibitive, especially in studies that aim to describe a food web rather than the diet of one or a few consumers of interest. Statistical techniques with fewer assumptions regarding data structure may therefore be more practical for most studies of trophic relationships in benthic habitats.

Cluster-forming methods such as classification and regression trees (CART) and hierarchical clustering do not assume homogeneity of covariances or multivariate normal data, and more importantly, the number of independent variables (i.e. FAs) is not limited by sample size (Smith et al. 1997, Budge et al. 2006). CART uses FAs with the greatest variance to divide the samples into dichotomous groups. CART may be useful as an exploratory method because it does not require selection of a smaller subset of FAs; however, the algorithm may use FAs that are not biologically meaningful to create tree nodes (Budge et al. 2006). For example, 16:0 is ubiquitous because most organisms are capable of synthesizing it *de novo*, but its relative abundance varies among taxa. CART also does not provide any indication of overall relationships among samples (Budge et al. 2006). Hierarchical clustering uses the overall similarity between samples to either link them into increasingly large groups or divide them into dichotomous groups (Hair et al. 2006). It is similar to CART in that it is an exploratory method with few assumptions regarding data structure, but in hierarchical clustering the distances between groups in the resulting dendrogram represent their dissimilarity (Budge et al. 2006). Hierarchical clustering does not test whether groups are significantly different from each other; however, the similarity profile (SIMPROF) analysis in the PRIMER software package (Clarke & Gorley 2006) can be applied to test whether each node of the dendrogram represents a statistically significant division (Clarke et al. 2008).

Similarity-based statistical techniques are especially useful for FA data because they do not require homogeneity of covariances or multivariate normality, can be used with a large number of variables, and include a variety of both exploratory and hypothesis-testing methods (Clarke 1993). FA data are often used in similarity-based analyses without transformation to avoid giving artificial weight to FAs that are present in small quantities (Howell et al. 2003,

Hall et al. 2006, Kelly et al. 2008, 2009), but in most cases, analyses on untransformed data effectively only use abundant FAs. The data are used to calculate a triangular matrix of similarities (such as Bray-Curtis similarity) between each pair of samples. MDS then uses the ranks of similarities in an iterative process to find the spatial arrangement of samples that best represents the relationships among them (Clarke & Green 1988, Hair et al. 2006). MDS is an exploratory analysis that does not take into account any *a priori* groups and so can be used to classify consumers with unknown diets (Howell et al. 2003, Guest et al. 2008) as well as to look for spatial (Hughes et al. 2005, Fernandez-Jover et al. 2007) and temporal (Wai et al. 2008) differences in overall consumer FA composition. It has also been used to analyze the results of controlled feeding studies, both for comparing overall FA composition of animals on different diets (Kelly et al. 2008, 2009) and for tracking changes to FA composition over time (Hall et al. 2006). While MDS provides a valuable visual representation of relationships among samples, an additional technique is necessary for hypothesis testing. Analysis of similarity (ANOSIM) is a non-parametric, similarity-based analogue to MANOVA that tests for differences in overall FA composition through permutations of the rank similarity matrix (Clarke 1993). ANOSIM is frequently used in conjunction with MDS, both for comparing *a priori* groups and for testing whether clusters observed in the MDS plot differ significantly from each other, and for performing pairwise comparisons among groups (Hughes et al. 2005, Hall et al. 2006, Kelly et al. 2008, 2009, Wai et al. 2008). The ANOSIM R statistic is a measure of similarity between groups on a scale of 0 to 1: values between 0.5 and 0.75 indicate that groups are different, but have some degree of overlap, while $R > 0.75$ indicates well-separated groups (e.g. Jaschinski et al. 2011). Large sample sizes in ANOSIM may result in significant p-values even when groups are not well-separated, so the R-value should be reported. However, ANOSIM is best used for 1-way tests (e.g. 1 species on different diets) and not for more complicated study designs (e.g. multiple species on different diets), as it does not calculate the proportion of variance attributable to each factor or test for interactive effects. Permutational multivariate analysis of variance (PERMANOVA) is a 'semi-parametric' MANOVA analogue that allows analysis of factorial and nested designs (Anderson 2001, 2005). PERMANOVA may therefore be better suited than ANOSIM for analysis of some FA data (e.g. Fernandez-Jover et al. 2007, Crawley et al. 2009, Kelly et al.

2012). ANOSIM, MDS, and PERMANOVA are available in the PRIMER software package (Clarke & Gorley 2006).

While it is important to understand relationships among the overall FA compositions of samples in any study, the individual FA driving overall patterns may also be of interest. In studies of benthic food webs, both the characteristic FAs of potential food sources and the FAs contributing most to differences among consumer species or feeding groups can be informative. SIMPER analysis measures similarity between each pair of samples and calculates average similarity within groups and dissimilarity between groups, and examines the contribution of each FA to average similarity (Clarke 1993, Clarke & Gorley 2006). SIMPER results can be used to identify potential indicator FAs. For example, 22:6n-3 had the highest contribution to dissimilarity between horse mackerel *Trachurus mediterraneus* collected from reference areas and near fish farms: reference fish were rich in 22:6n-3 as compared with fish associated with fish farms (Fernandez-Jover et al. 2007). Where FAs identified by SIMPER are known indicators of specific food sources, the results can indicate diet of consumers. In the previous example, 22:6n-3 was an indicator for juvenile fish and cephalopods, which were the likely prey of reference fish (Fernandez-Jover et al. 2007). In deep-sea sea stars, FA composition of suspension feeders was characterized by copepod indicator FA 20:1n-9 as well as the phytoplankton indicators 20:5n-3 and 22:6n-3, which Howell et al. (2003) also attributed to feeding on copepods.

Univariate statistics

Although multivariate statistical methods should be used to analyze most FA datasets, the additional use of univariate tests on tracer FAs is appropriate following a finding of significant differences among groups using multivariate procedures. Univariate procedures such as analysis of variance (ANOVA) are commonly used for testing whether the concentration of a single FA differs among groups of samples, and several such comparisons may be performed on a single dataset if α is adjusted (e.g. using Bonferroni correction). ANOVA is commonly used in field studies to compare levels of specific FAs in organic material or animals among sampling locations (Cook et al. 2000, Alfaro et al. 2006, Meziane et al. 2006), or seasons (Bachok et al. 2003, Meziane et al. 2006), and among consumer species (Kharlamenko et al. 2001, Alfaro et al. 2006, Meziane et al.

2006). However, there are often several possible sources for a given indicator FA found in a consumer, and ecological knowledge and common sense should be applied in their interpretation. If available, information from a controlled feeding study using the consumer of interest provides valuable insight as to which of the potential indicator FAs in the food items are actually conserved in consumer tissues (Cook et al. 2000). ANOVA is also appropriate for evaluating levels of indicator FAs in food and consumers in controlled feeding studies (Hall et al. 2006, Kelly et al. 2008, 2009). As with any statistical test, care should be taken to distinguish statistical significance from biological significance, as small within-group variance (especially common in controlled feeding studies) can produce a statistically significant result even when levels of the indicator are similar among groups (Budge et al. 2006).

COMBINING STABLE ISOTOPE AND FA ANALYSES

Stable isotope (SI) and FA analysis have similar advantages over estimating diet based on gut contents and direct observation, but the methods differ in their spatial variability and their ability to distinguish certain primary producers (Richoux & Froneman 2008, Guest et al. 2010). SI signatures of primary producers are mainly influenced by environmental factors such as light, temperature, and CO₂ availability, while FA signatures are also strongly influenced by physiological processes (Guest et al. 2010). SIs are well-suited to distinguishing among macroalgae and various classes of vascular plants (Peterson 1999), but in some cases they cannot be used to estimate bacterial contribution to a benthic food web because isotopic signatures of sediments tend to overlap with those of primary producers (Richoux & Froneman 2007, 2008). The carbon isotopic values of benthic diatoms also overlap with those of major macroalgal taxa, making their contribution to consumer diets difficult to estimate using SIs alone (Newell et al. 1995). FA analysis can easily distinguish bacteria and diatoms from other primary producers, and therefore is well-suited for characterizing benthic food webs which may have significant bacterial and microalgal components (Sargent et al. 1987, Kharlamenko et al. 2001, Richoux & Froneman 2008). Nitrogen isotopic ratios are enriched with each trophic transfer, which can be used to indicate the trophic level of consumers (Peterson 1999). This enrichment varies to some extent with diet and should be checked in controlled feeding studies (McCutchan et al. 2003), but cross-

validation between $\delta^{15}\text{N}$ and indicator FAs may prove useful for determining trophic level. The complementary characteristics of SI and FA analysis have led to an increasing number of studies using both tracers (Guest et al. 2010).

Bulk SI analysis with FA analysis

Bulk SI analysis (i.e. based on tissue samples as opposed to isolated compounds) is commonly used to complement FA analysis as a means of cross-validating results, particularly when 1 type of tracer is insufficient to separate all food sources. In an estuary in northern New Zealand, mangroves and seagrass were well separated using carbon isotope ratios, but the $\delta^{13}\text{C}$ values of most consumers fell between those of seagrass and brown algae, which were not well separated (Alfaro et al. 2006). Seagrass and brown algae had distinct indicator FAs, however, as did other food sources including diatoms and bacteria, so the presence of these indicator FAs in consumer tissues was used to identify their diets (Alfaro et al. 2006). In a temperate estuary in South Africa, the crab *Cleistostoma edwardsii* and gastropod *Haminoea alfredensis* both had highly enriched $\delta^{13}\text{C}$ values, suggesting seagrass, epibionts, or surface sediments as a major carbon source (Richoux & Froneman 2007). FA analyses in a complementary study identified high levels of the diatom indicator 20:5n-3 in these animals, indicating that epibionts were the most likely food source (Richoux & Froneman 2008). FA analysis of whelks *Thais clavigera* and bivalves *Saccostrea cucullata* and *Septifer virgatus* in nearshore shallow habitats in Hong Kong showed only that all 3 had similar levels of indicators for dinoflagellates and zooplankton, but carbon and nitrogen isotope ratios of food sources and consumers revealed that *T. clavigera* preys primarily on the suspension-feeding bivalves (Wai et al. 2008). Levels of the seagrass FA indicators 18:2n-6 and 18:3n-3 were relatively low in all consumers in a seagrass bed in the Sea of Japan (Kharlamenko et al. 2001), but these FAs are also precursors for PUFA synthesis, so FA results alone could not be used to conclusively rule out the importance of seagrass to these consumers. Carbon isotope ratios can be used to estimate the contribution of seagrass-derived carbon because seagrass tends to differ from other primary producers in its carbon isotopic composition (Kharlamenko et al. 2001, Alfaro et al. 2006). Carbon isotope ratios of consumers indicated that the majority of consumers derived at least half of their carbon from seagrass,

and that grazers consumed a combination of seagrass and epiphytes (Kharlamenko et al. 2001). In a seagrass bed in the Baltic Sea, $\delta^{13}\text{C}$ values of seagrass overlapped with those of epiphytes, but were distinct from those of sand microflora and a red macroalga (Jaschinski et al. 2011). Because the level of the seagrass indicator FA 18:4n-3 was low in all consumers, seagrass was considered an unlikely food source and was removed from the SI mixing model. Based on indicator FAs and SI data, diets of most mesograzers appeared to shift from epiphytes to sand microflora when epiphytes were scarce (Jaschinski et al. 2011).

Because FA tracers can be ambiguous with regard to a consumer's trophic level, nitrogen isotopes are a particularly valuable source of additional information in FA studies of food webs (Richoux & Froneman 2007, Guest et al. 2010, Jaschinski et al. 2011). The FA composition of the polychaete *Perenereis nuntia vallata* in a temperate estuary in South Africa contained high levels of PUFAs and diatom indicators, as well as elevated bacterial FA, which taken alone would likely indicate surface deposit feeding (Richoux & Froneman 2008). However, the $\delta^{15}\text{N}$ signature was also enriched, suggesting that *P. nuntia vallata* also consumes animal material and may obtain some of its tracer FA by consuming other deposit feeders (Richoux & Froneman 2007). Both the $\delta^{15}\text{N}$ signature and abundance of the carnivory indicator FA 18:1n-9 were seasonally enriched in the omnivorous isopod *Idotea baltica*, suggesting that *I. baltica* consumes more animal material when epiphytes are scarce (Jaschinski et al. 2011). Sea urchins *Strongylocentrotus droebachiensis* in low-productivity habitats offshore from kelp beds had more enriched $\delta^{15}\text{N}$ signatures with increasing distance from the kelp bed, suggesting that they consumed more animal material as the availability of drift kelp decreased (Kelly et al. 2012). FAs identified as indicators for kelp (18:1n-9, 18:4n-3, 20:4n-6) were present in sea urchins at all sites and distances from the kelp bed, but there was no detectable spatial pattern in their relative abundance.

SIs of indicator FAs

Compound-specific SI analysis of individual FAs can help to discern trophic pathways with greater resolution than is possible with bulk SI analysis or FA analysis alone. Pond et al. (2002) used $\delta^{13}\text{C}$ ratios of indicator FAs to investigate the source of PUFAs in deep-sea hydrothermal vent worms. In the tube-worm *Ridgea piscesae*, $\delta^{13}\text{C}$ values of 16:1n-7 and

18:1n-7 indicated a chemosynthetic origin for these FAs, which is consistent with the reliance of *R. piscesae* on endosymbiotic bacteria (Pond et al. 2002). However, $\delta^{13}\text{C}$ values for 20:4n-6 and 20:5n-3 were closer to expected values for photosynthetic microplankton. Similarly, in the mixotrophic tubeworm *Protis hydrothermica*, $\delta^{13}\text{C}$ values for 20:5n-3 were consistent with a chemosynthetic origin, but values for 22:6n-3 suggested a photosynthetic origin. Although other studies have found PUFA synthesis by bacteria (Jøstensen & Landfald 1997), these results indicate that chemosynthetic bacteria are not the only source of PUFAs for *R. piscesae* and *P. hydrothermica*, and that other sources, including synthesis by the tubeworms, should be investigated (Pond et al. 2002). Jack et al. (2009) compared diets of rock lobster *Jasus edwardsii* in 3 marine reserves in New Zealand using bulk SI analysis and $\delta^{13}\text{C}$ values of selected indicator FAs. Bulk SI analysis revealed that rock lobsters in 2 of the 3 reserves used suspended particulate matter and macroalgae as their basal carbon sources, suggesting that they preyed on the abundant filter-feeding mussel *Mytilus edulis galloprovincialis*. Carbon and nitrogen isotope signatures of rock lobsters from the third reserve suggested that carbon from terrestrial detritus or chemoautotrophic bacteria contributed significantly to their diets, and elevated 16:1n-7 indicated the presence of either diatoms or bacteria. The $\delta^{13}\text{C}$ value for 16:1n-7 from these lobsters was within the expected range for sulfur-oxidizing bacteria, and the authors concluded that the symbiont-bearing clam *Solemya parkinsonii* is the most likely food source for the rock lobsters in the third reserve. Ice algal diatoms in the Arctic were distinct from pelagic diatoms in their FA composition, but $\delta^{13}\text{C}$ values of the diatom tracers 16:4n-1 and 20:5n-3 enabled the use of mixing models to quantitatively estimate the contribution of each diatom source to higher trophic levels (Budge et al. 2008).

RECOMMENDATIONS AND DIRECTIONS FOR FUTURE RESEARCH

Both the complexity of benthic food webs and the ability of consumers to modify dietary FAs introduce sources of error to studies using FAs in benthic habitats, but the reliability of results can be improved by considering these factors when designing a study. An effort should be made to characterize the FA composition of as many likely food sources as possible so that indicator FAs in the consumer can be attributed to the correct source. Whenever possible, potential

food sources should be collected from the same habitat as the consumers, rather than using literature values from another habitat, because indicator levels are variable even among closely-related species, and a single FA may have several sources in some habitats. Ideally, the transfer of indicator FAs from food to consumer should be verified in controlled feeding experiments before indicators are used in field studies. Data from these experiments should be examined for evidence of FA biosynthesis and selective retention. The lack of unique FA markers for any species imparts uncertainty to the interpretation of FA data, such that inferences are always stronger when corroborated with data from direct observation, gut contents, or SI analysis. The use of FAs in field studies of benthic food webs should be limited to (1) classifying consumers into trophic groups, (2) identifying FAs associated with trophic groups, and (3) detecting unusual foods with unique or rare indicator FAs. These studies should be undertaken with attention to potentially confounding variation in FA composition with tissue type, reproductive status, and season.

As with most ecological methods, appropriate allocation of sampling effort and use of statistical techniques are important in studies using FA analysis. FAs of consumers may be highly variable among replicates, so relatively large sample sizes may be required to detect differences among groups. Similarity-based measures have relatively few constraints and are appropriate for most FA datasets. Univariate statistics can be used to test for differences in FA levels among groups, but these should be corrected for multiple comparisons. Because of the limited resolution of FA data and complex FA metabolism of consumers, the results of FA studies in benthic systems thus far have been qualitative. Quantitative estimates of contents of mixed diets using FA analysis are possible in seabirds and marine mammals, but these techniques require careful calibration even for consumers with limited ability to modify dietary FAs (Iverson et al. 2004) and may therefore be impractical for invertebrates.

The application of FA analysis to benthic food webs has revealed trophic relationships in habitats that are otherwise difficult or impossible to study, traced the fate of primary production through detrital and other pathways, and confirmed linkages that were previously detected using other methods. Benthic communities pose certain challenges for FA analysis in that they often contain many potential food sources, and consumers may be capable of modifying their dietary FAs. A balanced approach that includes field sampling and controlled feeding experiments, multivari-

ate and univariate analyses, and cross-validation with other methods will generally yield the best results.

Despite the limitations of FA analysis in benthic food webs, some studies suggest promising areas for further investigation. These include (1) measuring FA assimilation by invertebrates through comparison of tissue and fecal FA profiles in controlled feeding studies (Hall et al. 2006); (2) using indicator FAs to detect non-native food sources such as aquaculture waste (Cook et al. 2000); and (3) the use of species-specific dietary markers, such as NMI FAs for benthic invertebrates (Budge et al. 2007) or sterols for primary producers (Brown & Belt 2012), as these may provide more reliable results than either FA or SI analysis.

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Appendix 1. Summary of fatty acids (FAs) used as dietary tracers in selected studies of benthic food webs. Non-methylene-interrupted (NMI) FAs are those with double bonds not separated by a methyl group (CH₂); prefixes 'i' and 'ai' indicate a methyl branch at the second or third carbon from the terminal methyl group, respectively (i.e. branched FAs). MUFA: monounsaturated FA; PUFA: polyunsaturated FA; LCFA: long-chain FA

Sample	Dietary tracer FA	Location (depth)	Source
Deep sea			
Algal material	20:5n-3 + 22:6n-3	NE Pacific (4100 m)	Drazen et al. (2008a)
Animal material	18:1n-9 20:1 MUFA		
Copepods	22:1 MUFA		
Animal material	18:1n-9/18:1n-7 ratio > 1	NE Pacific (4100 m)	Drazen et al. (2008b)
Copepods	20:1 and 22:1 MUFA		
Bacteria	18:1n-7 Odd-chain and branched FA NMI FA	NE Atlantic (1053–4840 m)	Howell et al. (2003)
Copepods	22:1n-11 + 20:1n-9		
Deep-sea fishes and crustaceans	18:1n-9		
Dinoflagellates	22:6n-3		
Photosynthetic microplankton	PUFA		
Protozoa and microeukaryotes	20:4n-6		
Sediment	Saturated FA		
Deep hydrothermal vents			
Chemoautotrophic bacteria	16:1n-7, 18:1n-7	Juan de Fuca Ridge (2200 m)	Allen et al. (2001)
Methanotrophic bacteria	n-8 MUFA		
Bacteria	n-7 MUFA with low 20:5n-3 and 22:6n-3	East Pacific Rise and Galapagos Rise (2600 m)	Ben-Mlih et al. (1992)
Phytoplankton	16:4n-3 + 18:4n-3 + 18:3n-3		
Zooplankton	20:1n-9 + 22:1n-9		
Methanotrophic bacteria	16:1n-5, 16:1n-6, 16:1n-8, 18:1n-6, 18:1n-8	Mid-Atlantic Ridge (840–3600 m)	Colaço et al. (2007)
Phytoplankton	20:5n-3 + 20:6n-3		
Sulfate-reducing bacteria			
<i>Desulfobacter</i>	10Me16, cyc18:0		
<i>Desulfovibrio</i>	i17:1n-7, i15:1n-7, i19:1n-7		
Sulfur-oxidizing bacteria	16:1n-7, 18:1n-7 i17:1n-5, 18:1n-6		
Bacteria	16:2n-4, 18:2n-4	Mid-Atlantic Ridge	Pond et al. (1997)
Scavenging/necrophagy or phytodetritus	20:5n-3 + 22:6n-3		
Sulfur-oxidizing bacteria	16:1n-7, 18:1n-7	Juan de Fuca Ridge (2200 m) and East Pacific Rise (2600 m)	Pond et al. (2002)
Shallow hydrothermal vents			
Bacteria	Branched FA 18:1n-7	Kurile Islands	Kharlamenko et al. (1995)
Brown macroalgae	C18 + C20 PUFA		
Diatoms and bacteria	16:0/16:1n-7 < 1		
Diatoms and brown and red macroalgae	20:5n-3		
Arctic			
Carnivory	18:1n-9/18:1n-7 ratio > 1	Spitsbergen, NE	Graeve et al. (1997)
Copepods	20:1n-9 + 22:1n-11	Greenland, and Barents Sea (50–570 m)	
Diatoms	16:1n-7, 20:5n-3		
Benthic mollusks	High n-7/n-9 ratio	Alaska	Budge et al. (2007)
Antarctic			
Cryptophytes or haptophytes (microalgae)	18:4n-3	Weddell Sea and Bransfield Strait	Graeve et al. (2001)
Diatoms	16:1n-7, 20:5n-3		
Necrophagy	18:1n-9		
Copepods	20:1 and 22:1 MUFA	Weddell and Lazarev Seas (200–1400 m)	Hagen et al. (2000)

(continued overleaf)

Appendix 1 (continued)

Sample	Dietary tracer FA	Location (depth)	Source
Tropical estuaries			
Bacteria	$\Sigma 15 + \Sigma 17$ 18:1n-7	Northern New Zealand	Alfaro et al. (2006)
Brown macroalgae	18:1n-9		
Diatoms	20:5n-3 16:1/16:0 > 1.6 $\Sigma 16/\Sigma 18 > 2$ 20:5n-3/22:6n-3 > 1		
Dinoflagellates	22:6n-3 20:5n-3/22:6n-3 < 1		
Mangroves	LCFA (>24:0)		
Red macroalgae	20:5n-3/20:4n-6 > 10		
Seagrass	18:2n-6 + 18:3n-3		
Zooplankton	20:1 + 22:1		
Bacteria	Branched FA + 18:1n-7	Okinawa, Japan	Bachok et al. (2003)
Copepods	20:1n-9, 20:1n-11		
Diatoms	20:5n-3		
Dinoflagellates	22:6n-3, 18:4n-3		
Mangrove detritus	16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3		
Bacteria	i17:0, ai17:0 18:1n-7	Sea of Japan	Kharlamenko et al. (2001)
Diatoms	16:1n-7 20:5n-3		
Dinoflagellates, cryptomonads	18:4n-3		
Fungi, protozoa, algae	20:4n-3		
Seagrass	18:2n-6, 18:3n-3		
Zooplankton	22:6n-3		
Bacteria	i15:0, ai15:0, i17:0, ai17:0, 18:1n-7	Okinawa, Japan	Meziane & Tsuchiya (2000)
Diatoms	20:5n-3		
Green macroalgae	18:2n-6, 18:3n-6		
Vascular plants	26:0, 28:0, 30:0		
Bacteria	i15:0, ai15:0, i17:0, ai17:0, 18:1n-7	Queensland, Australia	Meziane et al. (2006)
Diatoms	20:5n-3		
Mangroves	LCFA (>24:0) 18:2n-6, 18:3n-3		
Microalgae	16:1n-7/16:0		
Temperate estuaries			
Bacteria	Branched FA + 18:1n-7	NW France	Meziane et al. (1997)
Diatoms	20:5n-3		
Salt marsh plants	LCFA (>24:0), 18:3n-3		
Bacteria	i15:0, ai15:0, i17:0, ai17:0	SE South Africa	Richoux & Froneman (2008)
Diatoms	20:5n-3, 16:1n-7 16:1/16:0		
Dinoflagellates	22:6n-3 22:6n-3/20:5n-3		
Salt marsh plants and green algae	18:1n-9		
Vascular plants	18:2n-6, 18:3n-3		
Animal material	18:1n-9	N Germany	Jaschinski et al. (2011)
Bacteria	18:1n-7		
Diatoms	16:1n-7, 20:5n-3		
Red algae	20:4n-6		
Seagrass	18:4n-3		
Rocky subtidal			
Kelp	20:4n-6	Nova Scotia, Canada	Takagi et al. (1980)
Diatoms	16:1n-7, 20:5n-3	Nova Scotia, Canada	Kelly et al. (2012)
Kelp	18:1n-9, 18:4n-3, 20:4n-6		
Kelp	20:4n-6, 18:4n-3	Scotland	Cook et al. (2000)