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FATTY ACID SIGNATURES DIFFERENTIATE MARINE MACROPHYTES AT ORDINAL AND FAMILY RANKS[1]

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Primary productivity by plants and algae is the fundamental source of energy in virtually all food webs. Furthermore, photosynthetic organisms are the sole source for ω-3 and ω-6 essential fatty acids (EFA) to upper trophic levels. Because animals cannot synthesize EFA, these molecules may be useful as trophic markers for tracking sources of primary production through food webs if different primary producer groups have different EFA signatures. We tested the hypothesis that different marine macrophyte groups have distinct fatty acid (FA) signatures by conducting a phylogenetic survey of 40 marine macrophytes (seaweeds and seagrasses) representing 36 families, 21 orders, and four phyla in the San Juan Archipelago, WA, USA. We used multivariate statistics to show that FA composition differed significantly ($P < 0.001$) among phyla, orders, and families using 44 FA and a subset of seven EFA ($P < 0.001$). A second analysis of published EFA data of 123 additional macrophytes confirmed that this pattern was robust on a global scale ($P < 0.001$). This phylogenetic differentiation of macrophyte taxa shows a clear relationship between macrophyte phylogeny and FA content and strongly suggests that FA signature analyses can offer a viable approach to clarifying fundamental questions about the contribution of different basal resources to food webs. Moreover, these results imply that taxa with commercially valuable EFA signatures will likely share such characteristics with other closely related taxa that have not yet been evaluated for FA content.

Key index words: **algal systematics; essential fatty acids; fatty acids; food web biomarkers; marine algae; marine macrophytes; primary production; seagrasses**

Abbreviations: **ALA, α-linolenic acid (18:3ω3); ARA, arachidonic acid (20:4ω6); DHA, docosahexaenoic acid (22:6ω3); EFA, essential fatty acids; EPA, eicosapentaenoic acid (20:5ω3); FA, fatty acids; FAME, fatty acid methyl esters; GC, gas chromatograph; GCMS, gas chromatography-mass spectrometry; LIN, linoleic acid (18:2ω6); MSI, multiple stable isotopes; PCA, principal components analysis; PERMANOVA, permutational multivariate analysis of variance; PERMDISP, permutational test of multivariate dispersions; SDA, stearidonic acid (18:4ω3); SI, stable isotope; SJA, San Juan Archipelago**

Photosynthetic organisms are the source of virtually all energy in food webs. Upper trophic level consumers are constrained by this production (e.g., Power 1992), but for many systems the relative importance of different sources of production to consumer communities is debated and poorly resolved (e.g., see Pace et al. 2004, Brett et al. 2009). In nearshore marine and aquatic environments, sources of primary production may be autochthonous [e.g., macrophytes (macroalgae and seagrasses), single-celled phytoplankton (diatoms, dinoflagellates)], or allochthonous (e.g., terrestrial plants). Assessing the relative importance of these distinct basal resources in marine food webs has been a complex problem because direct observation or gut content analysis is not possible for many primary consumers. The use of stable isotopes (SI; Duggins et al. 1989, Kaehler et al. 2000, Page et al. 2008) and fatty acids (FA; Budge et al. 2008, Richoux and Froneman 2008, Copeman et al. 2009) as biomarkers in this regard has shown promise but these approaches assume that all important food sources are identified, adequately characterized, and

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represented in mixing models. Moreover, FA and SI can only be used when all relevant primary production sources have distinct signatures, which is often not the case with SI. Herein, we investigated the phylogenetic differentiation of FA content in the four major macrophyte phyla found worldwide in nearshore marine habitats, to evaluate the potential of FA signature analysis for clarifying fundamental questions about energy sources in food webs.

FA are necessary constituents of the tissues of all living organisms. Because FA have distinct chemical structures, it is possible to routinely identify up to 70 FA within a given organism (Iverson 2009). The identities and quantities of FA in a given sample constitute the FA 'signature'. Of particular interest are the essential FA (EFA), generally defined as ω-3 and ω-6 FA families, which animals are unable to synthesize (Bell and Tocher 2009), and as such are potentially conservative molecular biomarkers. In addition, EFA are especially useful in a food web context because they are important for physiological processes (Sargent et al. 1999, Muller-Navarra 2008), including survival, growth, and reproduction in a wide range of aquatic species (Brett and Muller-Navarra 1997), but are only synthesized in biologically relevant amounts by plants and algae (Gladyshev et al. 2009). Previous research on the role of algae as a supply of EFA has focused on the contribution of EFA by phytoplankton to aquatic food webs (Kainz et al. 2004, Ravet et al. 2010).

The role of nearshore macrophytes as a subsidy (e.g., Polis et al. 1997) source of EFA for marine food webs is unknown. This is particularly surprising due to the high productivity (Mann 1973, Duarte and Cebrian 1996) and known importance of benthic algae for nearshore invertebrate assemblages (e.g., Dunton and Schell 1987). As little as 10% of this production is believed to be directly consumed by herbivores as standing stock (Mann 1988). The vast majority of this energy is exported as a spatial subsidy to subtidal (Duggins et al. 1989), intertidal (Rodriguez 2003), pelagic (Kaehler et al. 2006), and terrestrial (Polis and Hurd 1995) food webs. The patterns of FA composition of marine macrophytes may be used to further explain the role of macrophytes as a source of EFA to higher trophic levels and to increase the resolution of marine food web models if these patterns are conserved in consumers. The transfer of EFA synthesized by plants and algae to food webs should be of fundamental interest to resource managers, as these primary producers are the ultimate source of ω-3 and ω-6 EFA in higher trophic level consumers, which are harvested for human consumption.

Northeast (NE) Pacific nearshore marine macrophyte communities contain a very diverse mix of species (>640 taxa) representing four phyla (Gabrielson et al. 2006). The FA composition of <10% of these species is known. Algae encompass a wide diversity of organisms, often only distantly related to each other (Stengel et al. 2011), which are known to exhibit an astounding array of FA (Harwood and Guschina 2009), even among closely related taxa. Lang et al. (2011) recently demonstrated a significant phylogenetic signal in the FA composition of cultured microalgae, and several studies have reported on the FA content of marine macrophytes in different parts of the world (Khotimchenko 1998, Graeve et al. 2002, Khotimchenko et al. 2002, Hanson et al. 2010, Kumari et al. 2010). However, whether or not marine macrophytes segregate taxonomically with respect to their FA composition has not been demonstrated explicitly for a diverse assemblage of taxa.

We conducted a broad survey of the FA content of 40 NE Pacific marine macrophyte taxa representing 36 families, in 21 orders, across four phyla (Table 1; seagrasses, Anthophyta; brown algae, Ochrophyta; green algae, Chlorophyta; red algae, Rhodophyta) in the San Juan Archipelago (SJA), NE Pacific, to evaluate the taxonomic resolution of macrophytes as basal resources in a food web context. We compared this analysis with an evaluation of published macrophyte FA data (1994–2010) from an additional 123 independently collected taxa from 36 families, in 21 orders (Table S1 in supplementary material) across the same four phyla in all major oceans of the world. Specifically, we asked: (i) Does macrophyte FA composition differ among phylogenetic categories of phylum, order, or family level using 44 FA in the SJA dataset? (ii) Is the same taxonomic resolution achieved with the SJA dataset using only a subset of seven EFA? (iii) Are locally observed patterns consistent with published global macrophyte EFA data?

METHODS

NE Pacific macrophytes.

Selection of taxa: Our goal was to compare FA signatures from the four major marine macrophyte phyla present worldwide in nearshore waters. We selected species to maximize taxonomic diversity by creating a list of 80 macrophyte species that we expected to find during a May sampling period (see below) in the SJA. We removed species from the list that were difficult to identify due to lack of reliable morphological/anatomical traits (Gabrielson et al. 2006). The list was filtered to maximize the number of orders. Once an order was represented by one taxon, we selected multiple taxa within that order: (i) if each species was from a different family, (ii) if the different species could be found in different habitats (e.g., intertidal vs. subtidal) and (iii) in the case of Laminariales (kelps), we included eight species because of their biomass dominance in the drift (Britton-Simmons et al. 2009) in the SJA. Recent molecular work has shown that the green algal species commonly referred to as *Ulva lactuca* in the NE Pacific and other temperate marine waters in the northern and southern hemisphere has been misidentified (O'Kelly et al. 2010). We therefore extracted DNA from two specimens we had identified as *U. lactuca* and amplified the *rbc*L gene following the methods of O'Kelly et al. (2010). The sequences obtained match those from temperate-zone specimens that have been assigned to "*U. lactuca*." We refer to this entity as

TABLE 1. 40 NE Pacific marine macrophyte taxa studied in the San Juan Archipelago, USA.

Phylum	Order	Family	*Genus species*	Depth[a]	ID#[b]
Anthophyta	Alismatales	Zosteraceae	*Phyllospadix scouleri*	−3	1
			Zostera marina	−5	2
Chlorophyta	Bryopsidales	Codiaceae	*Codium fragile*	−2	3
	Cladophorales	Cladophoraceae	*Cladophora columbiana*	+2	4
	Prasiolales	Prasiolaceae	*Prasiola meridionalis*	+3	5
	Ulvales	Ulvaceae	*Ulva intestinalis*	+10	6
			Ulva sp.[c]	+2	7
Ochrophyta	Desmarestiales	Desmarestiaceae	*Desmarestia munda*	−6	8
	Dictyotales	Dictyotaceae	*Dictyota binghamiae*	−4	9
	Ectocarpales	Chordariaceae	*Soranthera ulvoidea*	0	10
		Scytosiphonaceae	*Scytosiphon lomentaria*	0	11
	Fucales	Fucaceae	*Fucus distichus*	+2	12
		Sargassaceae	*Sargassum muticum*	−4	13
	Laminariales	Alariaceae	*Alaria marginata*	0	14
		Costariaceae	*Agarum fimbriatum*	−8	15
			Costaria costata	−5	16
		Laminariaceae	*Nereocystis luetkeana*	−5	17
			Saccharina latissima	−6	18
			Saccharina sessilis	−2	19
			Saccharina subsimplex	−6	20
		Lessoniaceae	*Egregia menziesii*	−3	21
	Ralfsiales	Heterochordariaceae	*Analipus japonicus*	−3	22
	Syringodermatales	Syringodermataceae	*Syringoderma abyssicola*	−10	23
Rhodophyta	Bonnemaisoniales	Bonnemaisoniaceae	*Bonnemaisonia californica*	−3	24
	Ceramiales	Dasyaceae	*Rhodoptilum plumosum*	−6	25
		Delesseriaceae	*Polyneura latissima*	−3	26
		Rhodomelaceae	*Neorhodomela larix*	+1	27
			Osmundea spectabilis	−5	28
	Corallinales	Corallinaceae	*Calliarthron tuberculosum*	−3	29
	Erythropeltidales	Erythrotrichiaceae	*Smithora naiadum*	−4	30
	Gigartinales	Dumontiaceae	*Constantinea subulifera*	−2	31
		Endocladiaceae	*Endocladia muricata*	+2	32
		Furcellariaceae	*Opuntiella californica*	−6	33
		Gigartinaceae	*Chondracanthus exasperatus*	−6	34
			Mazzaella splendens	0	35
		Kallymeniaceae	*Callophyllis flabellulata*	−5	36
	Halymeniales	Halymeniaceae	*Prionitis sternbergii*	+2	37
	Palmariales	Palmariaceae	*Halosaccion glandiforme*	+4	38
	Plocamiales	Plocamiaceae	*Plocamium pacificum*	−6	39
	Rhodymeniales	Rhodymeniaceae	*Sparlingia pertusa*	−6	40

[a]Depth collected from in the field (m) relative to datum.
[b]Taxa ID # corresponds to numbering in PCA in Fig. 1.
[c]This entity is generally referred to as *Ulva lactuca* in the NE Pacific but was recently shown to be a tropical taxon (O'Kelly et al. 2010) and was therefore treated as *Ulva* sp. until a valid name is assigned.

Ulva sp. in Table 1 until a valid name for this species has been assigned. Finally, the brown alga *Syringoderma abyssicola* was added opportunistically after we encountered it in the field because it represented a rare order in the local flora.

Sample collection: Previous work has documented variation in macrophyte FA signature by season (Nelson et al. 2002), within-thallus location (Khotimchenko and Kulikova 2000), across different light and temperature regimes and with depth (Becker et al. 2010). Whether or not such variation is biologically significant in food webs is currently unexplored. To minimize variation due to these factors, we constrained specimen collection to a 3-week window (21 May–10 June, 2010) for all but two species that were not found until 16 June and 16 August 2010. Moreover, when possible we collected only sporophytes for taxa with heteromorphic life histories. An exception was made for the red alga *Opuntiella californica* because the gametophyte is a conspicuous upright and the sporophyte an uncommon subtidal crust. As the potential for within-species site/location variability was unknown, we constrained sample collection to five locations in the SJA within a 15 km radius. For any given species we tried to collect all specimens at one site. The vast majority of specimens were collected at three sites: Point Caution, San Juan Channel (61% of all taxa; 48.56°N, −123.01°W), Skipjack Island, Boundary Pass (17%; 48.73°N, −123.03°W), and Andrews Bay, Haro Strait (15%; 48.55°N, −123.17°W).

We collected five replicate specimens (>2 m apart) of each species from its median depth distribution (using SCUBA for subtidal species). Specimens were stored in flow-through sea tables (<8 h) until cleaned and frozen (−20°C). Our 40 species represented a diverse array of thallus morphologies. To constrain our sampling to functionally comparable areas, we focused our sampling in the center of the vegetative ''blade'' (or comparable portion) of each thallus. Meristematic, reproductive, stipe, and holdfast tissues were avoided. We cleaned specimens by brushing gently with a toothbrush under filtered seawater before collecting ~2 g wet weight from each replicate. Small thallus size of some taxa required pooling multiple thalli into one replicate. In these

cases, we still collected the material to be pooled from locations >2 m apart in the field. We selected only tissue that was healthy and not fouled by encrusting epibionts.

Fatty acid extraction: We extracted FA from three replicates within 7 months of collection and retained the remaining samples as vouchers. We lyophilized samples for 48 h, ground the dry material into a powder, and extracted lipids following Brett et al. (2009). Briefly, 10 mg of dry material was suspended in a 4:2:1 chloroform/methanol/water mixture, sonificated, vortexed, and centrifuged before removing the organic layer. This procedure was repeated three times, the organic extracts were then pooled, and evaporated to dryness under nitrogen. Samples were then transesterified in a 1:2 toluene/1% sulfuric acid in methanol mixture for 16 h in a 50°C water bath. After cooling, 2% $KHCO_3$ and hexane:diethyl ether (with BHT 0.01%) was added, and after vortexing and centrifugation, the upper phase was removed. A second addition of hexane:diethyl ether and subsequent extraction was pooled with the first. Solvent was then evaporated off the derivitized FA methyl esters (FAME) and re-suspended in 1.5 mL of hexane prior to GC analysis. FAME were analyzed with an HP 6958 gas chromatograph (GC) equipped with an auto sampler and flame-ionization detector using an Agilent DB-23 column (30 m, 0.25 mm diam., 0.15 um film), and 37-component FAME standards mix (Supelco™, Bellefonte, PA; Taipale et al. 2011) with a total run time of 85 min. We cross verified FAME identification in our chromatograms by running a subset of our samples through a GC at a lab that had previously verified the FAME found in our samples using GCMS. This procedure ultimately identified a total of 44 unique FA. Individual FA were expressed as a percentage of total FA mass.

Global taxa data gathering. We gathered EFA data from the literature published between 1994 and 2010 (Fleurence et al. 1994, Khotimchenko 1998, Graeve et al. 2002, Khotimchenko et al. 2002, Kelly et al. 2008, Richoux and Froneman 2008, Allan et al. 2010, Hanson et al. 2010, Kumari et al. 2010). One challenge with published FA data is that researchers may interpret certain peaks as representing different FA, depending upon the analysis method used (e.g., GC-flame-ionization detection vs. GCMS), and report quantitative results for only several to >60 FA. In addition, when mining literature data, it is not often possible to control for factors such as season and depth across studies. Finally, incorrect species identification and contamination from unwanted, associated microscopic epi- and endo-phytic taxa is a complicating factor for macroalgae in particular. For this reason we felt justified in selecting data that were collected and evaluated in a manner as consistent as possible with our approach. This dataset was not assumed to be a comprehensive list of all published macrophyte FA data, but rather a broad subsample of taxa from many regions sufficient for our research question. The global taxa list, sources, phylogenetic grouping variables, and sampling regions are presented in Table S1. Due to the plasticity of names in the algal literature, we searched in AlgaeBase (Guiry and Guiry 2011) at genus rank for each species from the literature and aligned each entity with its current order and family name. All literature FA values were analyzed with the a priori hypothesis that results of the global data analysis would not differ from what was observed in the SJA. From each paper, we used only the seven EFA (see below) that were previously used in the SJA analysis.

Data analysis. We used PERMANOVA (Anderson 2001) to evaluate differences among groups with multivariate FA datasets for both the SJA and global datasets. Taxonomic factors for both analyses were nested (e.g., family was nested with order, which was nested with phylum). We used PERMANOVA to test for the significance of the factors order and 10 ocean basin sub-regions (Arctic, Southern, N Indian, SE Indian, SW Indian, NE Pacific, NW Pacific, NE Atlantic, NW Atlantic, and SE Atlantic) and their interaction in the published global dataset. Because of assumed relationships among the taxa with regard to taxonomic ranking, factors were treated as fixed in analyses. PERMANOVA is a nonparametric analog to MANOVA where statistical significance is determined by repeated ($n = 9{,}999$) permutations of the raw Euclidian distance matrix to generate null distributions for comparing with observed values. We calculated percent variance in the PERMANOVA table by dividing the variance component estimated for each factor by the sum of all variance components to quantify the relative magnitude of effects (Hanson et al. 2010). PERMANOVA does not require multivariate normality, so the results reported herein are from running the analyses on untransformed FA data. However, because the routine can be sensitive to differences in-group dispersions (described below), we arcsine-transformed ($x' = \sin^{-1}\sqrt{x}$) the FA datasets and confirmed that the results of the analyses were not sensitive to a lack of transformation.

To investigate the FA variation of different lineages, we used PERMDISP (Anderson 2006) to test the null hypothesis of no differences among the dispersions of macrophyte FA signatures using grouping variables phylum and order in both the SJA full 44 FA dataset (40 taxa) and the combined SJA and global EFA dataset. In this pooled analysis only, one reported FA signature (selected randomly) was used to represent a taxon that multiple sources had evaluated in different regions so that more commonly evaluated taxa would not receive more weight in the analysis than rarely evaluated taxa. In addition, we later verified that the results of this analysis were not dependent upon the taxa selected. We used a post hoc pairwise PERMDISP of orders to investigate relative differences in FA dispersion between orders in the combined EFA dataset, including only those orders with at least 3 independent taxa. Results of this analysis were summarized by plotting the mean distances of the group centroid (±1 SE) value as calculated from the Euclidean distance matrix. We used Principal Components Analysis (PCA) ordinations of Euclidean resemblance matrices of non-transformed percent FA composition data for multivariate data visualization. Arcsine-transformations to the raw FA data did not affect visual interpretation of the PCA results. PCA plots were accompanied by eigenvector plots showing FA, which correlated (Pearson >0.4) with the first two principal components (PCs). The seven ω-3 and ω-6 EFA used in the reduced analyses were selected a-priori and included: 18:2ω6 [LIN], 18:3ω6, 18:3ω3 [ALA], 18:4ω3 [SDA], 20:4ω6 [ARA], 20:5ω3 [EPA], and 22:6ω3 [DHA]. Due to the large number of FA variables and taxa studied, we limited our attempt to summarize individual FA means and focus instead on evaluation of our research questions, and provided the entire SJA FA dataset as a supplementary table (see below). However, we did summarize the 5 abundant EFA for the SJA dataset at the phylum level. Two EFA, 18:3ω6 and DHA, were not included in this post hoc summary because they each accounted for a grand mean of less than 1% of total EFA in the SJA taxa. All analyses were performed using PRIMER v. 6.0 and PERMANOVA+ add on (PRIMER-E Ltd., Plymouth, UK).

RESULTS

San Juan Archipelago taxa. Using the full suite of all 44 routinely identified FA, we found that that macrophyte FA composition differed significantly among phylogenetic grouping variables of phylum, order, and family (PERMANOVA, $P = 0.0001$; Table 2, Fig. 1) for the 40 taxa evaluated. A second analysis of the SJA taxa using only seven ω-3 and ω-6 EFA (see Methods) yielded comparable results with the phylogenetic grouping variables of phylum,

order, and family still explaining significant variation among species (PERMANOVA, $P = 0.0001$; Table 2, Fig. 2). The comparisons across phyla explained the largest portion of the variability (Table 2); 36.5% in the 44 FA analysis and 40.5% in the EFA analysis. The order and family ranks explained progressively less variation than the phylum (Table 2), with the residual (i.e., within taxon) variances accounting for 13.9% and 13.2%, respectively.

The mean ± SD values of FA percent composition (three replicates per taxon except for *Syringoderma* where $n = 1$) for all 44 FA quantified in this analysis are presented in Supplementary Dataset S1 (see supplementary material). Multivariate data from the analyses of the 44 FA and 7 EFA datasets are visualized using PCA in Figs 1 and 2. PCA eigenvector plots (Figs S1, S2 in supplementary material) show the trajectory of the correlations (Pearson, >0.4) of the FA variables to PCs 1 and 2 in Figs 1 and 2, respectively. The multivariate dispersions of the FA variability (44 FA) of the SJA phyla and orders were not equal (PERMDISP; Phyla: $F = 20.319$, $df_1 = 3$, $df_2 = 2$, $P = 0.001$; Orders: $F = 13.652$, $df_1 = 20$, $df_2 = 97$, $P = 0.001$). A summary plot of five abundant EFA (see Methods) in the SJA taxa (Fig. 3) shows the mean percent composition (±1 SD) and discriminating potential of EFA for differentiating macrophyte phyla. The red algae were particularly characterized by the EFA ARA and EPA (highly variable means of ~11% and ~17% of total FA within this rank, respectively), 14:1ω5, and the saturated FA 16:0 and 18:0. The brown algae exhibited a relatively even distribution of EFA (see Fig. 3), sharing an abundance of ARA and EPA (~13% and ~12%, respectively) with the red algae, and were primarily separated from this group by a relative lack of 16:0 and 18:0 (Fig. S1). Green algae had relatively consistent abundance of the EFA LIN, ALA, and SDA (means of ~5%, 17%, and 7%, respectively). The Ulvales primarily drove this pattern in SDA in the green algae. Seagrasses were consistently segregated from other taxa by a concurrent and consistent abundance of the EFA LIN and ALA (means of ~7% and 46% of total FA, respectively). The saturated FA 20:0 and 24:0 were not very abundant (0–2% of total FA) in the seagrasses, brown and green algae, but were never found in red algae and thus were also useful in discriminating these groups (Fig. S1).

Published global taxa. Because taxonomic resolution at the order and family ranks was achieved using only EFA in the SJA dataset, we then mined published values of these same EFA from marine macrophyte studies worldwide (123 macrophyte taxa, 21 orders, and 36 families in 10 distinct ocean ''regions''; Table S1). We found the same

TABLE 2. Results of PERMANOVA analyses on three datasets testing for differences in FA percent composition of macrophyte taxa between phylogenetic grouping factors of phylum, order, and family. Dataset 1 uses all 44 identified FA in San Juan Archipelago (SJA) macrophyte taxa ($n = 40$; Table 1). Dataset 2 uses only seven ''essential'' ω-3 and ω-6 FA (EFA, see Methods) from the SJA taxa. Dataset 3 uses only the same seven EFA, gathered from macrophyte FA literature (n = 123; Table S1). The SJA dataset includes FA data for three replicates per taxon, whereas published data are mean taxon percent FA values (with varying levels of within group replication). Analyses use Type III sums of squares, fixed effects, and use 9999 permutations (see Methods). Percent variance (% Var) is the variance component estimated for each factor divided by the sum of all variance components to quantify the relative magnitude of effects.

Dataset					
Source	df	MS	Pseudo-F	*P* (perm)	% Var
1. SJA – 44 FA					
PHY	3	9,732	114.4	0.0001	36.5
ORD(PHY)	17	1,392	16.4	0.0001	25.3
FAM(ORD[PHY])	11	1,099	12.9	0.0001	24.2
Residual	86	85.1			13.9
Total	117				
2. SJA – 7 EFA					
PHY	3	7,083	156.2	0.0001	40.5
ORD(PHY)	17	719	15.9	0.0001	23.6
FAM(ORD[PHY])	11	574	12.7	0.0001	22.7
Residual	86	45.3			13.2
Total	117				
3. Global – 7 EFA					
PHY	3	4,532	27.4	0.0001	36.2
ORD(PHY)	19	670	4.1	0.0001	22.5
FAM(ORD[PHY])	16	313	1.9	0.0083	15.3
Residual	84	166			26.0
Total	122				

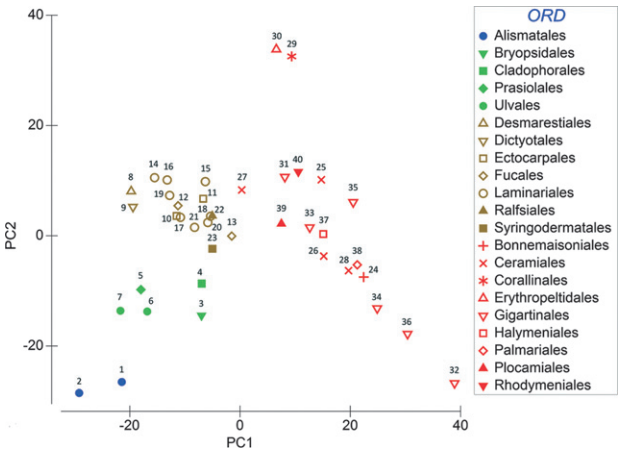

FIG. 1. Principal Component Analysis (PCA) visualization of mean multivariate macrophyte fatty acid (FA) composition data. PCA is run on a Euclidean distance matrix of mean percent composition of all 44 regularly identified FA for 40 San Juan Archipelago (NE Pacific) macrophyte taxa from 21 orders and 36 families. Symbol colors represent different phyla (Anthophyta, blue; Chlorophyta, green; Ochrophyta, brown; Rhodophyta, red) and phylogenetic order as symbols. The first two of five PCs (plotted herein) account for 65.2% of the cumulative variation and show the lowest two-dimensional solution of the dataset. Numbers above symbols correspond to the mean value of each taxon in the analysis (Table 1). A PCA eigenvector plot (Fig. S1) shows the trajectory of the correlations (Pearson, >0.4) of the FA variables to PCs 1 and 2.

taxonomic resolution in the global macrophyte data (not including any of the SJA taxa) using only the seven EFA (PERMANOVA, $P = 0.0001$, Table 2, Fig. 4). The FA most important for differentiating the global EFA dataset were ARA, EPA, LIN, and ALA, shown in the trajectory of the correlations (Pearson, >0.4) of the FA variables to PCs 1 and 2 in Fig. S3 (see supplementary material). These patterns were consistent with the SJA EFA dataset (Figs. 2, 4, S2, S3).

Combined SJA and global taxa. Using the combined SJA and global EFA datasets, ($n = 163$ taxa) we found a significant interaction between taxonomic order and ocean basin (PERMANOVA, $P = 0.0097$, Table S2 in supplementary material), indicating that macrophyte EFA content within orders also depended upon geographic location. The multivariate dispersions of the variability of phyla and orders of the combined SJA and global EFA datasets were not equal (PERMDISP; Phyla: $F = 7.768$, $df_1 = 3$, $df_2 = 117$, $P = 0.001$; Orders: $F = 3.725$, $df_1 = 25$, $df_2 = 95$, $P = 0.005$). A summary plot of the pairwise comparisons of the multivariate dispersions of phylogenetic order (Fig. S4 in supplementary material) shows the mean distance to group centroid (±1 SE) in Euclidean space for all orders, demonstrating that some orders, particularly in the red algae, had much higher variation in FA signatures than others.

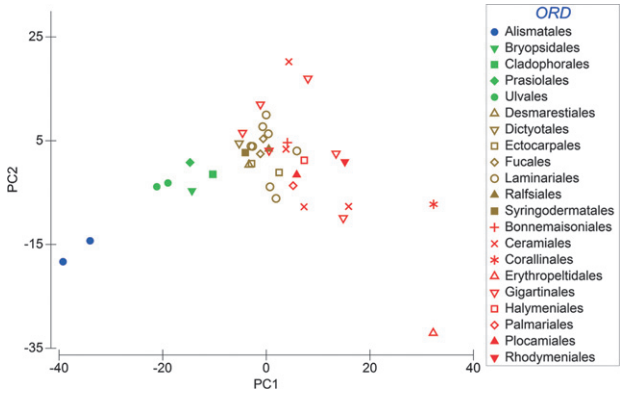

FIG. 2. Principal Component Analysis (PCA) visualization of mean multivariate macrophyte fatty acid (FA) composition data. PCA is run on a Euclidean distance matrix of mean percent composition of seven 'essential' ω-3 and ω-6 FA (selected a priori – see Methods) for 40 San Juan Archipelago (NE Pacific) macrophyte taxa from 21 orders and 36 families (Table 1). Symbol colors represent different phyla (described in Fig. 1) and phylogenetic order as symbols. The first two of five PCs (plotted herein) account for 73.0% of the cumulative variation. A PCA eigenvector plot (Fig. S2) shows the trajectory of the correlations of three of the EFA variables (Pearson, >0.4) to PCs 1 and 2.

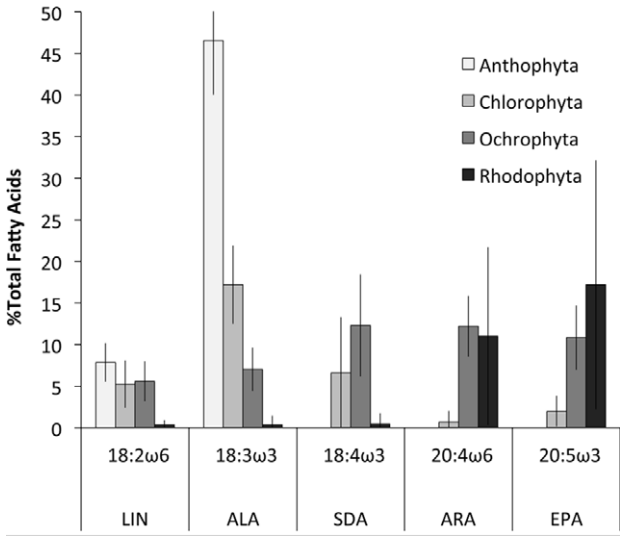

FIG. 3. Percent composition of five 'essential' ω-3 and ω-6 FA relative to the total FA in 40 macrophyte taxa evaluated from the San Juan Archipelago in the Anthophyta (empty), Chlorophyta (light gray), Ochrophyta (medium gray), and Rhodophyta (dark gray). This post hoc summary plot includes only the abundant EFA for the SJA dataset at the phylum level. Two EFA, 18:3ω6 and DHA, were not included because they each accounted for a grand mean of less than 1% of total EFA in the SJA taxa. Bars are means across all taxa in each division, error bars are SD.

DISCUSSION

We have shown that there is a substantial and clear taxonomic signal in macrophyte FA composition (Figs 1, 2, and 4). This taxonomic pattern was robust when the analysis was constrained to include only the seven EFA (Table 2, Fig. 4), which are only manufactured by plants and algae and are of key importance to animals in all food webs. Moreover,

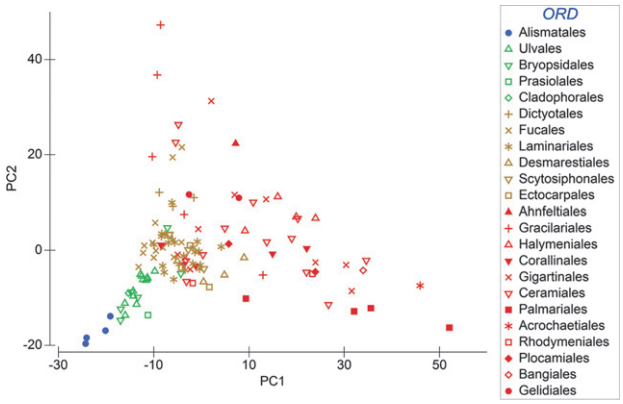

FIG. 4. PCA of global macrophyte FA composition data. PCA was run on a Euclidean distance matrix of the percent composition of only seven EFA reported for 123 global taxa from 21 orders and 36 families collected in 10 global ocean sub-regions (see Table S1). Symbol colors represent different phyla (described in Fig. 1) and phylogenetic order as symbols. Each plotted symbol represents EFA mean data for 1 published taxon, where taxa means in literature represent a range of independent specimens from 1->5. The first two of five PCs (plotted herein) account for 77.8% of the cumulative variation. A PCA eigenvector plot (Fig. S3) shows the trajectory of the correlations of four of the EFA variables (Pearson, >0.4) to PCs 1 and 2.

the taxonomic resolution of macrophyte EFA described herein was consistent across two separate datasets (40 SJA taxa and 123 global taxa) collected in different regions using different methods. Since 1972, a link between FA composition and marine macrophyte groups has been hypothesized (Jamieson and Reid 1972) and more recently discussed (Graeve et al. 2002), but only in this decade have researchers attempted to evaluate the these differences using statistical tests (e.g., Hanson et al. 2010, Kumari et al. 2010). However, implications from previous analyses have been limited by a relatively small number of taxa (eight macroalgal species and three seagrasses) of limited taxonomic breadth (no green algae) in the former (Hanson et al. 2010) and repeated univariate tests in the latter (Kumari et al. 2010). Our results offer strong multivariate statistical support for the previously hypothesized link between macrophyte phylogeny and FA content.

The differences in EFA composition among the three macroalgal groups (red, green, and brown algae) is perhaps not so surprising, as the evolutionary lineages of reds and greens diverged hundreds of millions of years ago and brown algae are unrelated to that lineage (Keeling et al. 2005). However, green algae and seagrasses are in the same lineage with the same suite of photosynthetic pigments and similar biochemical pathways, yet their FA also were divergent. The most likely explanation is that marine green algae have occupied that habitat for hundreds of millions of years, whereas seagrasses are recent colonizers of the marine environment. Both lineages have closely related freshwater and terrestrial members that warrant additional study to determine if the correlation we demonstrated is more widespread. Differences in FA signatures of microalgae between phylogenetic phyla, classes, and even within genera have recently been demonstrated for a large collection of cultured microalgae (Lang et al. 2011). Our taxa list did not allow for statistically meaningful within genera comparisons for the macrophytes.

EFA of the red algae were clearly more variable relative to the brown algae (Figs 2–4, S4). The brown and red algae in both datasets exhibit substantial variation in thallus morphology, yet morphological characters often do not reflect taxonomic similarity. Compare for example the brown algal orders Ectocarpales, which have generally 'simple' thallus morphology and isomorphic life histories, with Laminariales, which have large complex thalli and heteromorphic life histories. Despite these morphological differences, these are recognized as sister clades (Phillips et al. 2008), and their FA were very similar (Fig. 1). The large dispersion in the EFA of the well sampled orders of Gigartinales and Ceremiales (red algae) relative to the Laminariales (Fig. S4) may be related to the evolutionary history of the two lineages (Graeve et al. 2002). DNA evidence strongly supports that the red algae obtained their plastids through an ancient primary endosymbiotic event, whereas the lineage that includes brown algae obtained their plastids through a primary as well as a secondary endosymbiosis (Keeling 2004). The mechanism for the greater variety of the EFA found in Gigartinales, Corallinales, and Gracilariales compared to other red algal orders is currently unresolved and merits additional investigation.

Our FA analyses have important implications for food web research. Researchers often cite data from outside their own study system when defining 'FA biomarkers' without investigating the validity of those biomarkers for a particular question or region. In addition, it is not uncommon that one FA will be cited as a biomarker for an entire taxonomic group [e.g., 20:4ω6 (ARA) as a brown algal FA biomarker; Hanson et al. 2010]. Although we would not dispute that ARA is a common constituent and important FA in brown algae, we found this FA in similar amounts in some red algae (Fig. 3). Whereas the FA signatures of the macrophyte orders and families are clearly distinct, we also found that the factors oceanic region and order showed a significant interaction ($P < 0.01$, Table S2). This result may be in due to variation in the sampling season for the global taxa, or increased degree of FA unsaturation reported in algae in polar regions as compared to temperate analogs (e.g., Graeve et al. 2002). Although it is unclear what is causing the interaction of order and ocean region, this result supports the importance of evaluating potential primary producer FA biomarkers from the study system in question (Dalsgaard et al. 2003). Moreover, our analyses suggest that multiple FA, rather than individual FA, should be used concurrently as trophic markers.

We have shown that FA signatures of macrophyte groups differ to at least the rank of family, whereas SI signatures have been demonstrated to differ only to the rank of phylum. Recent work by Marconi et al. (2011) found wide heterogeneity in both $\delta^{13}C$ and $\delta^{15}N$ signatures for a comprehensive list of 85 macrophyte taxa in 4 phyla. Hanson et al. (2010) showed taxonomic differentiation at the phylum and species levels for $\delta^{13}C$, but not for $\delta^{15}N$ for a macrophyte assemblage, of 11 taxa. As researchers increase the number of elements considered in SI analyses (e.g., sulfur; Connolly et al. 2004), resolution power for this approach may increase. Additional research is needed to investigate whether or not MSI (i.e., >3 SI), applied to phylogenetically broad taxa lists, can provide the family level taxonomic resolution for marine macrophytes demonstrated herein.

Researchers are increasingly utilizing a combination of MSI and FA biomarkers for evaluating questions about the relative contribution of basal resources to food webs (e.g., Turner and Rooker 2006, Budge et al. 2008, El-Sabaawi et al. 2010), and

whereas the combination of both approaches may increase taxonomic resolution, it may not be feasible to use both approaches when faced with limited resources. In our experience, the raw materials costs of SI and FA analyses are relatively similar, but the FA extraction and quantification process is more time consuming and requires additional equipment and training. This added expense may be reasonable if the research question requires a fine level of taxonomic resolution (e.g., family or ordinal) of primary producers. However, both methods currently suffer from a lack of experimental evidence demonstrating the predicted fractionation in SI (e.g., Gannes et al. 1997, del Rio et al. 2009, but see Wehi and Hicks 2010) and transfer of FA biomarkers up the food chain in controlled feeding trials (but see Hall et al. 2006, Kelly et al. 2008, 2009). Such experimental feeding trials are clearly the most important future direction for MSI and FA trophic ecology and represent a crucial next step in evaluating whether or not the taxonomic differences in FA signature of primary producers actually transfer to upper trophic level consumers in predictable ways.

That phylum, order, and family level taxonomic resolution were elucidated using only a suite of seven ''essential'' ω-3 and ω-6 EFA will be of particular interest to ecologists who wish to use FA signature analysis for tracing primary producer contributions to food webs. Although the content of these EFA can be modified by animals through FA chain modification, animals do not have desaturase enzymes necessary to insert double bonds at the ω-3 and ω-6 positions of FA to synthesize these molecules *de novo* (Dalsgaard et al. 2003). It is important to account for the abilities of the consumers under study to elongate or retroconvert these EFA in controlled feeding trials (Hall et al. 2006, Kelly et al. 2009) before making quantitative assessments of various sources to consumer diets. Such feeding trials can take advantage of the phylogenetic rank differences in macrophyte FA signatures shown herein to evaluate the degree to which FA remain intact in the herbivore or are converted to other FA.

The response in consumer FA signatures to diets of different FA signatures is poorly understood or completely unknown for most consumer taxa. Despite this uncertainty, it is not uncommon for researchers to use published algae FA biomarkers as evidence that consumers are foraging on specific primary producers in the field (Richoux and Froneman 2008, Allan et al. 2010, El-Sabaawi et al. 2010). It may ultimately be possible to model consumer diets using quantitative FA signature analysis (QFASA; Iverson et al. 2004, Iverson 2009), however, such modeling requires a comprehensive dataset of FA signatures of all of the important/potential prey items and also requires knowledge about the consumers' ability to synthesize/modify FA (i.e., to account for potentially bioactive FA molecules in the consumer). Furthermore, such quantitative modeling will require additional evaluation of the potential importance of seasonal and geographic variation in primary producer biochemical signals. Herein we argue that researchers must first test the fundamental assumption that the FA composition of a diverse array of possible macrophyte basal resources actually differ before attempting to quantitatively or qualitatively model the transfer of these resources to consumers. Until now, this assumption has not been evaluated. This work further advances the goal of utilizing a quantitative approach like QFASA for an herbivorous consumer because it has helped identify the FA signatures of a wide range of potential consumers ''prey'' items.

We have shown that the four major marine macrophyte groups have distinct signatures of FA and even of EFA. Because EFA are limiting in many aquatic ecosystems (Brett and Muller-Navarra 1997, Litzow et al. 2006) and are synthesized only by plants and algae, they may function as conservative trophic tracers. The phylogenetic differentiation in EFA content of marine macrophytes offers finer taxonomic resolution (e.g., order and family ranks), than what has previously been demonstrated for SI (phylum rank) and FA. The fact that different macrophyte groups have distinct EFA signatures is also important in the context of ecosystem services, as large amounts of detached, drift macrophyte biomass is transported to deep subtidal (e.g., Britton-Simmons et al. 2012) and intertidal (e.g., Bustamante et al. 1995) habitats and is utilized for energy by consumers. The patterns of macrophyte EFA content are of particular interest in this context because animals rely upon the service of primary producers to synthesize and provide these molecules to animals that cannot generate ω-3 and ω-6 EFA *de novo*. In addition, the phylogenetic signal present in macrophyte FA signatures has important implications for commercial interests that seek to find and isolate valuable bioactive compounds such as EFA from the natural environment. The results presented herein show support for the hypothesis that taxa with potentially economically desirable FA signatures will share those characteristics with other closely related taxa that have not yet been evaluated for FA content. This taxonomic resolution may be used to address fundamental ecological questions about the relative importance of different basal resources to herbivorous consumers and marine food webs if controlled feeding trials can demonstrate predictable transfer of biomarker FA from distinct producers to primary consumers.

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Supplementary Material

The following supplementary material is available for this article:

Data S1. 40 macrophyte taxa studied in the San Juan Archipelago (NE Pacific, USA).

Fig. S1. PCA eigenvector plot overlaid onto Fig. 1 showing the trajectory of the correlations (Pearson, >0.4) of the FA variables to PCs 1 and 2.

Fig. S2. PCA eigenvector plot overlaid onto Fig. 2.

Fig. S3. PCA eigenvector plot overlaid onto Fig. 4.

Fig. S4. Summary plot of the post hoc pairwise comparisons of the multivariate FA dispersions of macrophyte phylogenetic order.

Table S1. 123 global macrophyte taxa, collection ocean region, and source (see text for references).

Table S2. Results of PERMANOVA testing for the significance of the factors order and ocean and their interaction in FA percent composition of 163 macrophyte taxa in 27 orders and 10 ocean sub-regions (Arctic, Southern, N Indian, SE Indian, SW Indian, NE Pacific, NW Pacific, NE Atlantic, NW Atlantic, and SE Atlantic).

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