Vol. 478: 1–14, 2013
doi: 10.3354/meps10310

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published March 25

OPEN ACCESS

FEATURE ARTICLE

Addressing assumptions: variation in stable isotopes and fatty acids of marine macrophytes can confound conclusions of food web studies

Megan N. Dethier[1,2,*], Elizabeth Sosik[3], Aaron W. E. Galloway[2,3], David O. Duggins[2], Charles A. Simenstad[3]

[1]Biology Department and [2]Friday Harbor Labs, University of Washington, Friday Harbor, Washington 98250, USA
[3]School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington 98195, USA

ABSTRACT: Studies that use biomarkers to elucidate consumer diets often must assume that these signatures are relatively invariant in space and time. We tested this assumption for multiple stable isotopes (MSI: $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) and fatty acids in 10 marine macrophytes (macroalgae and seagrass) on 3 different dates, and also quantified MSI at 3 sites in the coastal northeast Pacific. For all comparisons, we found significant variation in biomarkers among species, sites, and dates; furthermore, there were always significant site × species and date × species interaction terms, indicating that biomarkers do not change consistently across species among dates or sites. Despite this variation within and among species, biomarkers could readily distinguish macrophyte phyla. To observe how variation could affect conclusions about diets, we used a Bayesian mixing model to evaluate scenarios for a theoretical consumer given diverse diets, and with varying assumptions about the way it integrates foods over sites and seasons. Accuracy of the model runs (predicted diet versus simulated diet) increased with the number of biomarker variables, and depended strongly on initial assumptions about diets. The lowest accuracy occurred when biomarker values were based on macrophytes sampled only from 1 season but the consumer integrated foods across multiple seasons, a situation commonly seen in biomarker literature. Contrary to the notion that natural biomarker variation reduces insight into food web structure, exploring the potential mechanisms behind this variation should provide a more realistic view of coastal ecosystem dynamics.

KEY WORDS: Biomarker · Macrophyte · Variation · Isotope · Fatty acid · Seasonal

Biomarker signatures in intertidal and subtidal macroalgae and seagrasses may vary considerably in space and time.

Photo: Aaron Galloway

INTRODUCTION

The last decade has seen a growing body of literature in which biomarkers such as stable isotopes and fatty acids (FAs) are applied to diverse questions about the foundation, structure, and dynamics of aquatic food webs. Applications of both types of markers rely on assumptions that primary producers have relatively consistent 'signatures' that can be traced up through the trophic levels because these signatures either do not change when incorporated

*Email: mdethier@uw.edu

into consumers, or they change in relatively predictable ways (e.g. Peterson 1999, Dalsgaard et al. 2003, Fry 2006). This literature often glosses over implicit assumptions that are now coming under greater scrutiny, such as the degree of fractionation of stable isotopes in higher consumers (Mill et al. 2007, Wyatt et al. 2010, Bond & Diamond 2011), the consistency in FA signatures among related species of primary producers (e.g. Hanson et al. 2010, Galloway et al. 2012), and the amount of variation in space and time in stable isotopes (Page et al. 2008, Schaal et al. 2010, Woodland et al. 2012, Vinagre et al. 2012, Hyndes et al. 2013) and FAs (e.g. Nelson et al. 2002, Guest et al. 2010). For example, Guest et al. (2010) recently demonstrated that both multiple stable isotopes (MSI) and FAs vary to different degrees among replicate individuals, sites, and regions. Similarly, Boecklen et al. (2011) cautioned against overreliance on limited and static literature values.

A fundamental concern in these biomarker applications is whether natural variation will drive a signal-to-noise ratio beyond the capacity to differentiate food sources (Peterson & Fry 1987, Finlay et al. 1999, Ramos & González-Solís 2012). Alternatively, we can exploit natural variation and apply it to ecological questions and hypotheses; this requires both documenting the scope and scale of that variation and understanding the potential underlying mechanisms (Holst Hansen et al. 2012, Ishikawa et al. 2012). Our broader study is testing the subsidy of organic matter in macrophyte (algae and seagrass) detritus from productive shallow-water beds to deep basins below the photic zone; to investigate these processes, we first had to document the variation in macrophyte biomarkers. Variation in MSI values of marine macrophytes is poorly understood, but can occur at all scales. Spatial $\delta^{13}C$ variation can be significant at the scale of a single blade of a macrophyte (Stephenson et al. 1984, Fenton & Ritz 1989), due to differences among tissue types in factors such as growth rates, physiological status, and chemical composition. At larger scales, variation can also be significant among sites (Page et al. 2008, Guest et al. 2010, Schaal et al. 2010). These differences may relate to nutrient levels and other characteristics of the surrounding seawater (Holst Hansen et al. 2012), or to the thickness of the diffusion boundary layer formed by the local hydrodynamic environment, which may control productivity and the kinetics of carbon fractionation (Simenstad et al. 1993). Puzzlingly, spatial variation may sometimes be more significant over local than over regional scales, and some species may not display spatial variation at all (Simenstad et al. 1993, Guest et al. 2010). Macrophyte MSI values also vary temporally among seasons and years (Stephenson et al. 1984, Simenstad et al. 1993, Brenchley et al. 1997, Page et al. 2008, Schaal et al. 2010), with samples from colder seasons typically being depleted in $\delta^{13}C$ values compared to other seasons; however, not all species change consistently even within a given habitat (Hyndes et al. 2013). Temporal variation may stem from a number of factors, including seasonal freshwater discharges of varying inorganic carbon sources, or changes in macrophyte growth rates (Simenstad & Wissmar 1985, Wiencke & Fischer 1990, Hemminga & Mateo 1996, Carvalho et al. 2009). Of the studies exploring the multilevel controls on isotopic fractionation, the majority have looked only at $\delta^{13}C$, and only a few have considered both $\delta^{13}C$ and $\delta^{15}N$. To our knowledge, no other study has included $\delta^{34}S$.

The extent of variation in FA signatures of free-living marine macrophytes at regional and seasonal scales is not well known. However, it is generally accepted that FA biosynthesis occurs in the chloroplast and is subject to environmental conditions that affect photosynthesis such as light (Floreto & Teshima 1998), temperature (Becker et al. 2010), availability of nutrients (Floreto et al. 1996), and salinity (summarized by Guschina & Harwood 2006). There may be a general increase in the FA unsaturation (e.g. an increase in long-chain polyunsaturated FAs such as 20:5ω3 [EPA]; Honya et al. 1994) with decreasing regional temperature. Guest et al. (2010) found significant differences in the FA signatures of the brown algae *Phyllospora* (Fucales) and *Ecklonia* (Laminariales) at both local and regional scales. While several authors have documented seasonal variation in macrophyte FAs (e.g. Honya et al. 1994, Nelson et al. 2002), these differences have not been evaluated statistically or for ecological relevance. For example, it is unknown to what degree such patterns are consistent across a taxonomically diverse macrophyte assemblage and whether seasonal and geographic variation in macrophyte FA signatures is large enough to alter interpretation of food web models.

Here we expanded on the work of Guest et al. (2010) and Hyndes et al. (2013) to investigate spatial and temporal variation in MSI (carbon, nitrogen, and sulfur) and temporal variation in FAs. We used our own macrophyte data to evaluate the sensitivity of Bayesian mixing model results for simulated food transfers to a theoretical consumer when source biomarkers vary among seasons and sites. We also evaluated the number of biomarkers needed to accu-

rately reconstruct known diets. We confined our data analyses to patterns of variation in marine macrophytes. Biomarker signatures of microalgae, particulate organic matter, and consumers are still under investigation.

MATERIALS AND METHODS

Sites and sampling

Our study area is the San Juan Archipelago in the northern part of the Puget Trough, Washington state, USA (Fig. 1). The area is an inland sea protected from outer coast swells, but experiences wind-driven waves during fall and winter storms (Eckman et al. 2003). The marine basins among islands are bathymetrically complex, with steep-sided bedrock channels that transition to deep basins of mostly unconsolidated sediments (Britton-Simmons et al. 2012). The oceanography around the archipelago is well studied but also complex (Thomson 1994); the dominant hydrodynamic forces are tidally generated currents that occur at all depths and can exceed 100 cm s^{-1} (Eckman et al. 2003, Britton-Simmons et al. 2008). The Fraser River, ~45 km to the north in Canada, discharges large volumes of fresh water annually, with peak discharge in June and July; this discharge impacts the northern islands in the archipelago before becoming well mixed by the tidal currents in the narrow channels among the islands. The southern islands are primarily influenced by oceanic water masses from the Strait of Juan de Fuca. Thus, while the archipelago is relatively small, the complex circulation of different water masses is likely responsible for site-to-site differences in physical parameters (temperature, salinity, nutrients, sediment load) that can influence marine macrophyte productivity and physiology.

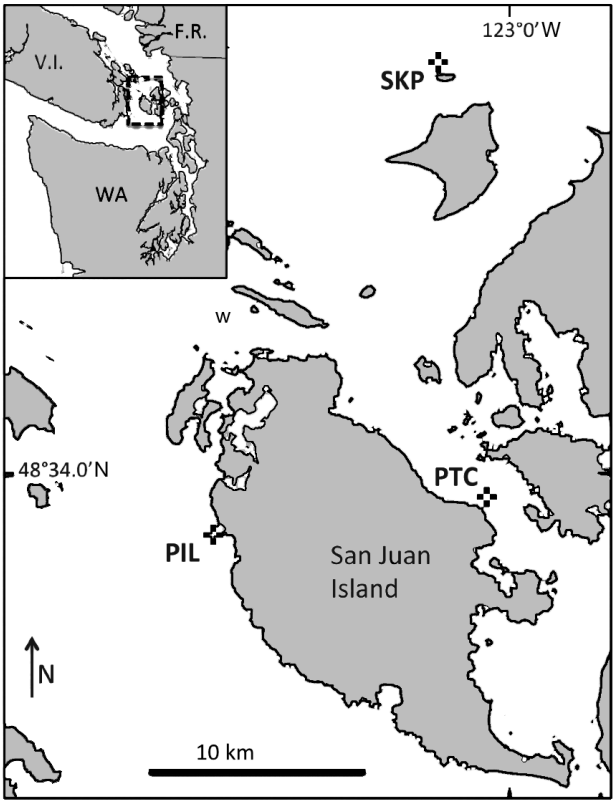

Fig. 1. San Juan Island in the western San Juan Archipelago and the 3 study sites where samples were collected for stable isotope comparisons. The sites cover a gradient of exposure to terrestrial freshwater inputs from the Fraser River to the north (FR; see inset), from Skipjack Island (SKP) to the higher salinity open ocean to the far west (Pillar; PIL) with Pt. Caution (PTC) in the San Juan Channel as intermediate salinity between the other 2 sites. Fatty acid samples were collected only at PTC

We quantified biomarkers from 3 replicate specimens of macrophytes collected (>2 m apart in the intertidal or via SCUBA) on 1 to 3 dates from their median depth distribution at 1 to 3 study sites (Fig. 1). Macrophytes collected were diverse (Table 1), including 4 kelp species (Order Laminariales), 5 other algae, and a seagrass. MSI and FA analyses are time consuming and expensive so we were unable to complete a balanced design for all sites, species, and dates (Table 1). Specimens were stored in flow-through sea tables for <8 h until cleaned and frozen at −20°C. To constrain our analyses to functionally comparable areas of macrophytes with different vegetative and reproductive morphologies (e.g. fertile portions scattered through the thallus versus in distinct sori), we sampled from the center of the vegetative portion of each thallus. Meristematic, reproductive, and stipe/holdfast/root tissues were avoided. We cleaned specimens of encrusting organisms by brushing gently with a toothbrush under filtered seawater before collecting ~2 g wet weight from each replicate. We selected only tissue that was healthy and free of epibionts.

Extractions

All MSI analyses followed methods employed in previous studies (Howe & Simenstad 2007, 2011, Page et al. 2008). Briefly, algal and seagrass tissues were rinsed in a 10% HCl solution and then with deionized water, except in cases where the effect of

Table 1. Summary of collection information for multiple stable isotope (MSI: $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) and fatty acid (FA) analyses conducted on marine macrophyte taxa in the San Juan Archipelago. PTC: Pt. Caution (see Fig. 1), which had the most complete sampling (species and dates). Additional sites were sampled only for MSI and only in August 2010

Phylum	Order	Species	May 2010 FA: PTC MSI: PTC	August 2010 FA: PTC MSI: 3 sites	March 2011 FA: PTC MSI: PTC
Anthophyta	Alismatales	*Zostera marina*	FA, MSI	FA, MSI	FA, MSI
Chlorophyta	Ulvales	*Ulva* sp.[a]	FA, MSI	FA, MSI	–
Ochrophyta	Desmarestiales	*Desmarestia munda*	FA, MSI	FA, MSI	–
	Fucales	*Fucus distichus*	FA, MSI	FA, MSI	–
	Laminariales	*Alaria marginata*	FA, MSI	MSI	–
		Agarum fimbriatum	FA, MSI	FA, MSI	FA, MSI
		Nereocystis luetkeana	FA, MSI	FA, MSI	FA, MSI
		Saccharina subsimplex	FA, MSI	FA, MSI	FA, MSI
Rhodophyta	Ceramiales	*Neorhodomela larix*	FA, MSI	FA, MSI	–
	Gigartinales	*Opuntiella californica*	FA, MSI	FA, MSI	–

[a]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 is therefore treated as *Ulva* sp. until a valid name is assigned

acid washing was tested (rinsed with de-ionized water alone). Acid washing was done to remove calcareous epibionts that may not have been completely removed by gentle brushing. Samples were freeze dried and ground to a fine powder using a modified dental mill (Howe & Simenstad 2007), then weighed using a microbalance and enclosed in tin capsules for analysis at Washington State University's Stable Isotope Core lab. Because FA samples were not acidified and we wanted to directly compare MSI and FA analyses from paired samples, we processed tissues of 4 macrophytes with and without acid-washing to compare its effects on MSI values.

We investigated temporal variation in FAs from a subset of the material collected (1 site only: Table 1). Following the methods employed by Galloway et al. (2012), we performed a total FA lipid extraction (modified Folch) from 10 mg of dry, ground material in a 4:2:1 chloroform:methanol:water mixture. This mixture was sonificated, vortexed, and centrifuged before removing the organic layer. This procedure was repeated 3 times, and the resulting pooled extracts were evaporated to dryness under nitrogen and transesterified in a 1:2 toluene:1% sulfuric acid in methanol mixture for 16 h in a 50°C water bath. Transesterified organic extracts were derivitized into FA methyl esters (FAMEs) and re-suspended in 1.5 ml of hexane prior to gas chromatography (GC) analysis. FAMEs were analyzed with an HP 6958 GC equipped with an auto sampler and flame-ionization detector using a 30 m Agilent DB-23 column, and 37-component FAME standards mix (Supelco™) with an 85 min run time (Taipale et al. 2011). We cross-verified our FAME identification of rare FAs (i.e. not in standards) with a GC from a second laboratory that had previously verified the identity of these FAs using GCMS. This procedure ultimately identified a total of 45 FAs. Individual FAs are expressed as a percentage of total FA mass. Because of their value as conservative biomarkers for primary producers in food web studies, many of our analyses were conducted using only the 6 most common ω3 and ω6 'essential' FAs (EFAs). These EFAs cannot be synthesized de novo by animals (Bell & Tocher 2009). Additional research into the ability of different animals to alter short-chain ω3 and ω6 precursor FAs into the longer chain EFAs is needed but is not specifically addressed here. The EFAs used in analyses here are: 18:2ω6 (LIN), 18:3ω6, 18:3ω3 (ALA), 18:4ω3 (SDA), 20:4ω6 (ARA), and 20:5ω3 (EPA).

Analytical approach

We analyzed multivariate biomarker data using a variety of routines in PRIMER v6 (Clarke & Gorley 2006) and permutational multivariate analysis of variance (PERMANOVA; Anderson et al. 2008). MSI and EFA data were analyzed separately and then combined for datasets where both biomarkers were analyzed. For the MSI and the combined datasets, biomarker variables were normalized (for each datum, subtracting the mean and dividing by the SD: Clarke & Gorley 2006) to put them all on comparable measurement scales. Resemblance matrices were calculated on untransformed data using

Euclidean distances. Patterns were visualized in non-metric multidimensional scaling (nMDS) plots. PERMANOVAs tested for the significance of differences in the suite of biomarkers among species, sites, and dates (all factors fixed). ANOSIM tests (2-factor crossed: species by site or species by date) were used to examine the biomarker similarities within versus among groups (e.g. species or dates), and an R statistic was calculated to indicate the degree of discrimination among groups. SIMPER routines on all datasets (e.g. FAs for all species on different dates) examined the role of individual biomarkers in contributing to the separation of different groups (species or dates). For full MSI and FA datasets, we used a CLUSTER analysis and SIMPROF test to find 'natural groupings' in the biomarker data and then examined to what extent these corresponded with taxonomic groups (species). A RELATE routine tested the relationship between similarity matrices of the MSI and FA, i.e. the degree to which among-sample similarities agreed for these biomarkers.

Model comparisons

FAs versus MSI

Eleven different diets for a theoretical herbivore, dubbed 'Snurchin,' were generated using MSI and FA data from macrophyte samples collected in August 2010 at the Pt. Caution site. For each food source, we calculated the average and standard deviation for each MSI and FA predictor variable. Ten of the simulated diets featured a different macrophyte as the dominant component (contributing between 30 and 50%), and contributions from each other macrophyte species were randomly assigned (between 0 and 30%) for each diet. An additional diet featured equal contributions of each species (~11%). All diet contributions summed to 100%. These proportions reflect the varied feeding opportunities for local consumers; Britton-Simmons et al. (2009) found that the greatest single contributor to subtidal drift algae comprised only 37% of the total drift available. The diverse sources used in our model are therefore realistic for the probable complexity of herbivore diets. In addition, E. Sosik & C.A. Simenstad (unpubl.) found that modeling errors that occur when input data incorporate insufficient variance for a source become more pronounced as that source contribution increases. The consumer was assigned a set of fractionation values for each stable isotope predictor variable: for C = 0.8 ± 0.09‰; for N = 3.4 ± 0.10‰; and for S = 0.5 ± 0.31‰ (McCutchan et al. 2003, Yokoyama et al. 2005). The MSI signature for each individual was calculated by using each diet to create a weighted average MSI signature of all potential food items, after which fractionation values were added. By adding or subtracting the standard deviation of macrophyte isotope values to mean values, as well as adding or subtracting standard deviation in fractionation rates to mean fractionation rates (McCutchan et al. 2003, Yokoyama et al. 2005), a total of 9 different values for Snurchin 'samples' were created for each of the 11 diets to simulate realistic consumer variability.

The FA and MSI values of Snurchin were then entered into the Bayesian mixing model, Stable Isotope Analysis in R (SIAR), which was chosen because it is considered to be robust to unquantified sources of error (Jackson et al. 2009, Parnell et al. 2010). FA values were incorporated into the model as additional biomarkers, much in the same way that additional isotopes would be added, except that FA variables were not assigned fractionation values due to a lack of appropriate published estimates. The mixing model was run under the following conditions for each diet, using (1) only the MSI of the consumer and food sources; (2) only the EFAs of the consumer and food sources; (3) combined MSI and EFAs of the consumer and food sources; and (4) combined MSI, EFAs, and 19 non-essential FAs. Each model run generated probability distributions for the contribution of each food source to the consumer's diet. The predictions for each model configuration were based on the median value of the probability distribution for each macrophyte source. Median values were used to reduce the influence of skewed probability distributions and to better capture the central tendency of the predictions, although we acknowledge that this index of the most frequent solutions does not necessarily capture the most probable ones (Fry 2013). The diet predictions were evaluated for accuracy using a Bray-Curtis similarity index on the compiled median predicted diets from the model versus the compiled generated diets used to construct the simulated consumer. Each of the 4 model conditions was evaluated following this approach. We also evaluated model performance using the residual error term provided by SIAR, which predicts whether unknown sources contribute to consumer diets. Because all sources are known in these simulations, the proportion of the diet assigned to unknown sources in the predictions may be attributed to model inaccuracy.

Table 2. Parameters that define the 8 scenarios incorporating different degrees of spatial and temporal variation in food source biomarker data into modeled diets for a theoretical herbivore, 'Snurchin.' Each scenario uses all macrophyte biomarker variables (multiple stable isotopes, essential and and non-essential fatty acids) and evaluates all 11 simulated Snurchin 'diets' (see 'Materials and methods'). The final column lists the similarity between predicted and actual diets (see 'Results')

Scenario	Spatial and temporal sampling methods		Consumer diet		Accuracy
	Season of food-source sampling	Site of food-source sampling	Food available to consumer (season)	Food available to consumer (site)	Bray-Curtis similarity (%)
1	August	1 site	August	1 site	97.3
2	Multiple seasons and sites collected, all samples averaged		All sites and seasons		92.6
3	Multiple seasons and sites collected, all samples averaged		August	All sites	81.6
4	Multiple seasons	Multiple sites, averaged by season	August	All sites	95.3
5	Multiple seasons	Multiple sites, averaged by season	Multiple seasons	All sites	95.1
6	Multiple seasons	Multiple sites, averaged by season	August	1 site	90.4
7	August	Multiple sites, averaged	Multiple seasons	All sites	72.4
8	August	Multiple sites, averaged	May	All sites	63.3

Spatial and temporal variability

The effect of spatial and temporal variability was examined in a second model simulation by comparing the mixing model predictions from 11 different Snurchin diets using all macrophyte biomarker data (MSI, EFAs, and non-EFAs) under 8 different scenarios (Table 2). Each scenario accounted for varying degrees of actual spatial and temporal variability found in our macrophyte data. Consumer data for each diet and each scenario were generated as described in the previous section, but used means and standard deviations from subsets of the macrophyte data based on season and site. In the first 6 scenarios, we used different ways of incorporating real temporal and spatial variation into the model methods, and evaluated how they performed against the generated temporal and spatial variability of the Snurchin diets (Table 2). Consumer diet compositions may vary spatially due to consumer mobility and to movement of detritus in the system. Consumer biomarkers may vary temporally as dietary signatures are integrated at different rates during different seasons or parts of the consumer's life history. For simplicity, the model parameters refer only to the geographic extent and number of seasons that the consumer biomarkers may integrate from. In the final 2 scenarios, we examined the robustness of sampling regimes (sites and seasons) frequently found in food web literature to violations of assumptions about seasonal dynamics and biomarker integration rates. The accuracy of the predicted diets under each set of assumptions was again evaluated using a Bray-Curtis similarity index on the median predicted diets from the model versus the generated diets used to construct the simulated consumer.

RESULTS

Acid washing

Acid-washing had no effect on MSI values in the 4 macrophytes tested (seagrass and 3 kelp species: PERMANOVA 1, df = 34, pseudo-F = 0.38, Monte-Carlo p = 0.62). Thus, we subsequently used non-acid-washed samples for tests involving both FA and MSI analyses.

MSI variation with site and date

We compared MSI signatures of 9 macroalgal species plus the seagrass *Zostera,* all collected in August 2010 at 3 sites (Fig. 1). Detailed MSI data for all samples are given in Table S1 in the supplement at www.int-res.com/articles/suppl/m478p001_supp.pdf. For most species, replicate individuals had similar MSI signatures. Macrophyte MSI differed significantly among species but also among sites, and there was a significant site × species interaction (Table 3A, Fig. 2). The species effect was stronger (see R-values, Table 3A), suggesting that the suite of MSI values is powerful for differentiating many species, but the site effect and interaction show that for many macrophytes the same species from different sites cannot be expected to have statistically identical MSI values. Some species (e.g. *Opuntiella*, lower left Fig. 2) were very consistent among sites, while others (e.g. *Neorhodomela*) had very high dispersions among sites. A cluster analysis on the full dataset (Fig. 2) resulted in 2 clusters differing at $p < 0.05$; 1 cluster contained some of the *Zostera* samples (in the lower right corner: some replicates from 2 sites), and the second

Table 3. Results of PERMANOVA. Global R-values (see 'Materials and methods') were generated from 2-factor crossed ANOSIM tests. See Table 1 for abbreviations, sites, and dates. The 6 essential FAs (EFAs) included in these analyses are listed in 'Materials and methods: Extractions'. Analyses with only 1 date (A, F–H) are from May 2010; comparisons of 2 dates (C, D) are May and August 2010. Analyses with only 1 site (B–H) are from Pt. Caution. 10-species analyses (A, F–H) include all taxa in Table 1; 9-species (C, D) exclude *Alaria*; 4 species (B, E) are *Zostera, Agarum, Nereocystis,* and *Saccharina*. p (Monte Carlo) = 0.001 for each analysis

	Analysis	Factor	df	MS	Pseudo-*F*	Global R
A.	MSI 3 sites × 10 species	Species	9	20.15	72.4	0.835
		Site	2	3.31	11.9	0.421
		Species × Site	18	1.46	5.2	
B.	MSI 3 dates × 4 species	Date	2	22.5	42.6	0.808
		Species	3	17.4	32.9	0.705
		Date × Species	5	2.2	4.2	
C.	MSI 2 dates × 9 species	Date	1	5.8	20.1	0.575
		Species	8	11.3	39.3	0.814
		Date × Species	8	1.4	5.0	
D.	EFA 2 dates × 9 species	Date	1	778	58.23	0.733
		Species	8	1817	135.9	0.925
		Date × Species	8	171	12.77	
E.	EFA 3 dates × 4 species	Date	2	405	25.04	0.745
		Species	3	3756	232.2	0.770
		Date × Species	6	178	11.02	
F.	MSI 1 date, 10 species	Species	9	7.59	9.20	0.671
G.	EFA 1 date, 10 species	Species	9	1253	104.2	0.974
H.	MSI + EFA, 10 species	Species	9	25.32	19.91	0.966

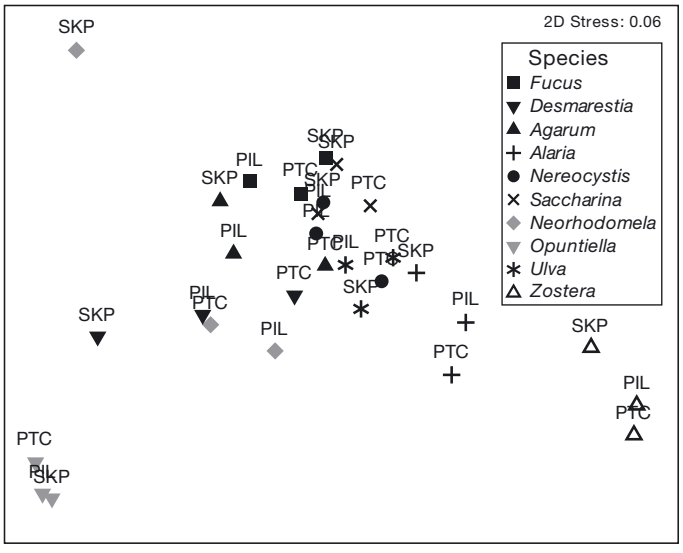

Fig. 2. Multidimensional scaling (MDS) plot for multiple stable isotopes (MSI: $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) for 10 macrophyte species from 3 sites (August 2010 data). Average values among 3 replicates per species are plotted for clarity, but analyses were run using all replicates. Site codes are given in Fig. 1. Macrophyte species are listed in full in Table 1. Black symbols are brown algae, grey symbols are red algae

contained all other species and samples. When the highly variable red alga *Neorhodomela* was removed to better distinguish patterns among the other species, a SIMPROF test recognized 5 clusters (not shown): (1) *Opuntiella* from all 3 sites; (2) some *Zostera* replicates from 2 sites; (3) the rest of the *Zostera* samples and some *Alaria* samples; (4) some of the *Desmarestia* samples; and (5) all other samples including the remaining 5 species. Thus, except for *Opuntiella*, MSI cannot distinguish among species when samples from multiple sites are analyzed together because values of replicates both within species and at different sites are too variable to distinguish.

MSI values of the 3 kelps and *Zostera* collected in March, May, and August 2010 at Pt. Caution (Table 1) differed significantly among species but even more markedly among dates (Table 3B). A SIMPROF test recognized 4 clusters: (1) all *Zostera* samples except for 2 August replicates; (2) the 2 August *Zostera* replicates; (3) all kelps in March; and (4) all kelps in May and August. Thus, MSI can distinguish kelps from *Zostera* regardless of date, but the MSI of both types of macrophytes varied substantially among dates. Particular isotopes varied highly among species in March (late winter), while values in May and August showed considerable overlap for most species and isotopes (Fig. 3). All 4 species, especially *Zostera,* showed gradually increasing $\delta^{13}C$ enrichment from early spring to late summer. Much (SIMPER analyses: 56%) of the March to May difference was driven by late-spring increases in $\delta^{15}N$ enrichment (Fig. 3). *Zostera* was also enriched in $\delta^{34}S$, but this was not evident in the other taxa. May to August changes were characterized primarily (SIMPER: 54%) by $\delta^{34}S$ depletion, although this pattern is driven by a large depletion in *Zostera* that was not observed in the kelps (Fig. 3). Overall, the 3 kelp species were rather similar in isotopic ratios and shifted similarly among dates, whereas *Zostera* was isotopically distinct, with its relatively enriched $\delta^{13}C$ and depleted $\delta^{15}N$ and $\delta^{34}S$.

An additional analysis of temporal variation was possible with a larger group of species for only 2 dates (May and August), including 2 additional Ochrophyta (not kelps), 1 Chlorophyta, and 2 Rhodophyta (including all species listed in Table 1 except for the kelp *Alaria*, which could not be collected on 1 date). Species were significantly different but there was considerable overlap in isotopic signatures among dates, although both factors and their interaction were again significant (PERMANOVA; Table 3C). The red alga *Opuntiella* was particularly different from other species, with very deplete $\delta^{13}C$ values in both seasons, and *Zostera* also stood out with enriched $\delta^{34}S$ values, especially in August (Table S1 in the supplement). Overall, the multivariate change from May to August was driven (SIMPER: 51%) by $\delta^{13}C$ enrichment.

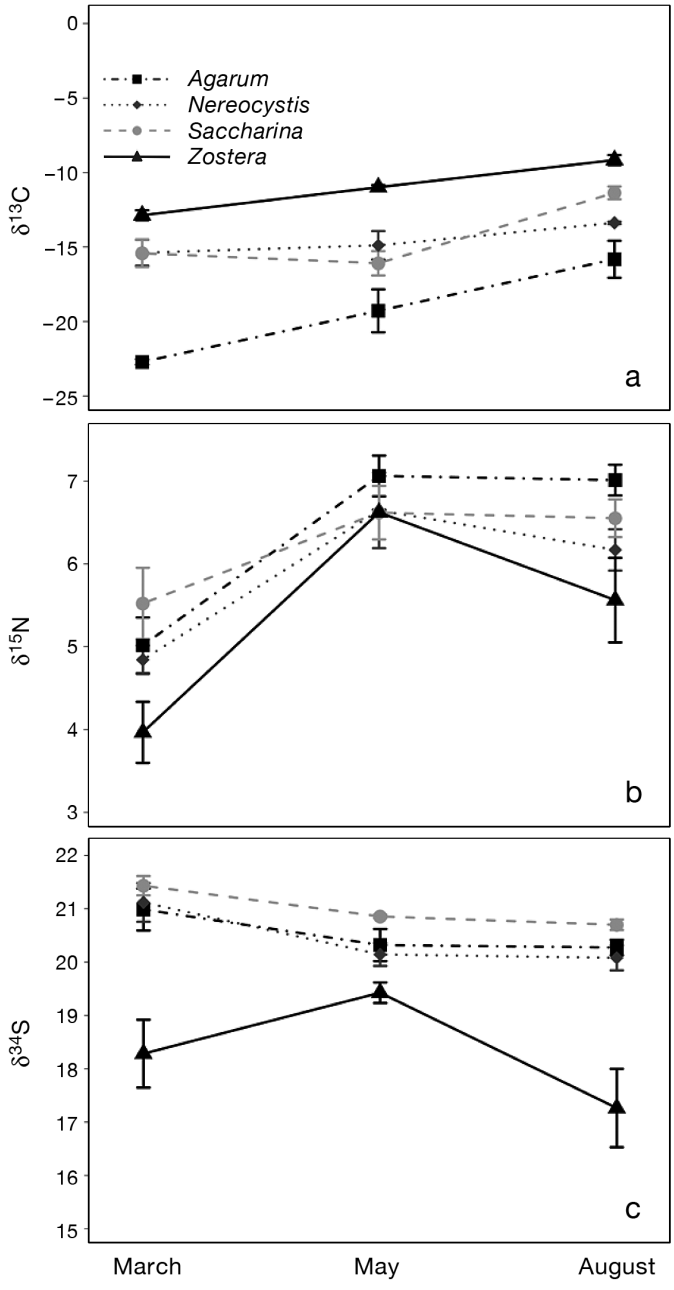

Fig. 3. Stable isotope ratios from 4 macrophyte species (3 kelps plus 1 seagrass) sampled on 3 dates in 2010 at 1 site (Pt. Caution). Values are means of 3 replicates ±1 SE

EFA variation with date

Detailed EFA data for all samples are given in Table S2 in the supplement. Comparison of the 9 taxa collected in May and August (at Pt. Caution; Table 1) indicated that EFAs were significantly different among both dates and species, as was the interaction term (Fig. 4, see Table 3D for PERMANOVA results). As with the MSI data, some species (e.g. *Zostera*) were highly variable among replicates and dates, while others (e.g. *Opuntiella*) were much more consistent. A cluster analysis distinguished 11 groups (Fig. 4). Only 2 species, *Opuntiella* and *Ulva*, had unique EFA signatures that were consistent among dates. *Zostera* and *Neorhodomela* had unique EFA signatures, but these changed significantly among dates (May and August samples are in different circled groups, Fig. 4). All of the brown algae (5 species) showed some degree of intermingling of signatures, either among species or among dates or both. Although variable in their contribution among species and between May and August, 5 FAs were especially important in distinguishing species (Table 4). The particular FA contributing the most to pairwise species dissimilarities was often (22 out of 36 comparisons) the same in May and August; in 18 of the comparisons, the top 3 FAs were all the same on the 2 dates.

Four species were sampled in March as well as May and August (Table 1). A PERMANOVA again found highly significant differences in FA composition among species, dates, and their interaction (Table 3E). *Zostera* EFAs from all 3 dates clustered together (not illustrated), driven by the dominance of 18:3ω3 (ALA) in these samples (see Table S2 in the supplement), although there was some variation among dates because of the virtual absence in March of its second most common FA, 18:2ω6 (LIN). Other unique clusters were *Saccharina* in March, *Agarum* in March, and *Agarum* in May; *Nereocystis* on all dates and all other kelps in August grouped in 2 other clusters. For the 3 kelps sampled in March, 20:5ω3 (EPA) was most abundant in that month, driving most of their differences among dates.

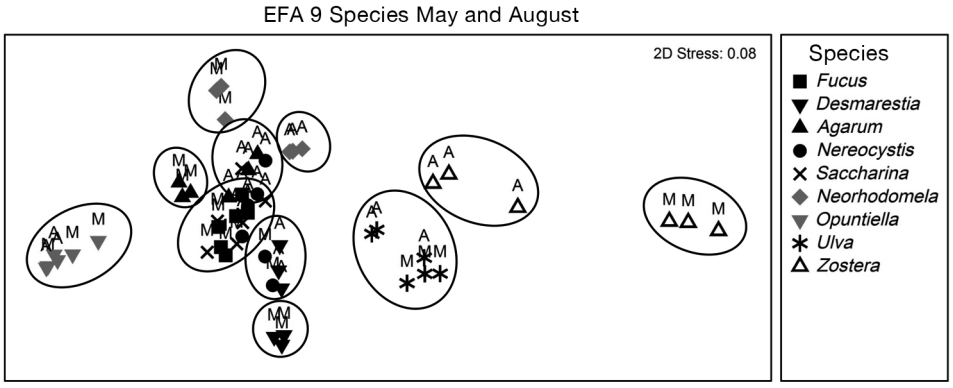

Fig. 4. MDS plot for essential fatty acids (EFAs) for 9 macrophyte species from Pt. Caution from May (M) and August (A) 2010. Each point is a replicate sample. Ellipses denote significantly different cluster groups (p = 0.05) identified in a SIMPROF analysis

Comparison of MSI and EFAs for differentiating macrophytes

To compare these 2 types of biomarkers for their ability to differentiate among primary producers, we analyzed tissues for both MSI and EFAs from the same replicates for 10 macrophyte species collected at 1 site (Pt. Caution) and date (May 2010). Both types allowed differentiation among species (significant species effects, Table 3F,G), although separation of species was much clearer for the EFAs (cf. Fig. 5A,B); this is probably a function both of the reduced dispersion among replicates within each species with EFA data and the fact that EFAs comprise 6 variables versus 3 for MSI. Based on PERMANOVA pairwise species comparisons, 17 of the 45 comparisons were not significant for MSI (i.e. the species could not be distinguished by their isotopic signatures), whereas only 3 of the pairwise comparisons were not significant using EFAs (all among brown algae). A RELATE analysis comparing the MSI and EFA resemblance matrices found that the relative differences among species were correlated (p = 0.003), but this relationship was not strong (ρ = 0.31). Combining the MSI and EFA data into 1 matrix, where each species is now described by all 9 variables, did not produce any stronger separation among species than the EFA data on its own (Table 3H). Of the 45 pairwise species comparisons, 5 were not significantly different with this combined dataset.

To evaluate our ability to chemically distinguish species regardless of date, we used both biomarkers to compare 9 species in May and August (Fig. 6; no FA data were available for *Alaria* in August). Significant clusters of samples were visible with both biomarkers. As with the FA data alone, few species had unique biomarker signatures that were consistent among dates (only *Opuntiella* and *Ulva*, Fig. 6). *Neorhodomela* again had biomarkers that differed significantly among dates, as did *Desmarestia.* One large cluster contained many (but not all) samples of 5 species of brown algae, and most *Zostera* replicates were in 1 group although 1 replicate was in a group of its own. Both FA and MSI contributed to cluster dissimilarity (SIMPER analyses), e.g. $\delta^{34}S$ distinguished clusters with *Opuntiella* and *Desmarestia*, and 18:3ω3 (ALA) distinguished most of the *Zostera*.

While only 2 of 9 species retained a distinct biomarker 'identity' among dates even when using 9 MSI and EFA variables (Fig. 6), our data suggest that phylum-level patterns of

Table 4. Individual fatty acids (FAs) that contributed the most to dissimilarities in FA composition among pairs of species in both May and August samples (from SIMPER analyses on individual dates). '%' is the average percent of the total FA composition on that date (variances are given in Tables S1 & S2 in the supplement at www.int-res.com/articles/suppl/m478p001_supp.pdf); 'Contrib.' is the percent that the FA contributed to dissimilarities among species on that date. *Indicates macrophytes that were in significantly different clusters in May and August, i.e. whose FA composition changed substantially among dates

Macrophyte	Key FAs	% in May	Contrib. to May	% in August	Contrib. to August
Opuntiella	20:4ω6	29.1	76	31.7	81
*Neorhodomela**	20:5ω3	21.1	29	3.1	86
	18:4ω3	4.5	39	0.9	1
Ulva	18:3ω3	22.4	67	18.7	65
	18:2ω6	4.1	9	5.5	28
Fucus	20:4ω6	14.4	11	10.1	19
	20:5ω3	8.9	10	4.8	33
*Desmarestia**	18:4ω3	20.9	2	14.3	54
	20:5ω3	12.2	46	8.4	21
*Agarum**	18:2ω6	9.7	5	6.6	29
	20:5ω3	13.8	64	4.7	24
Saccharina	18:4ω3	7.3	42	5.4	49
*Nereocystis**	20:4ω6	11.3	6	7.1	43
	18:4ω3	12.4	81	4.3	19
*Zostera**	18:3ω3	50.7	65	27.0	82
	18:2ω6	6.7	35	9.7	17

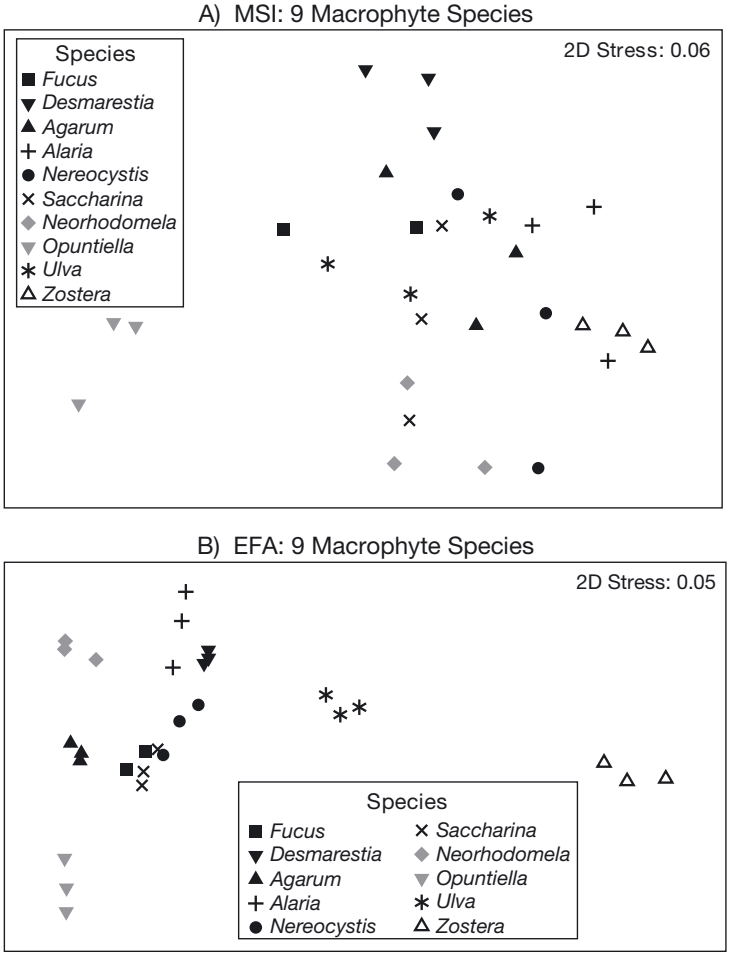

Fig. 5. MDS plots for (A) multiple stable isotopes (MSI: $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) and (B) 6 essential fatty acids (EFAs), each plot for 9 macrophyte species from 1 site and date (Pt Caution, May 2010). Each point is a replicate sample

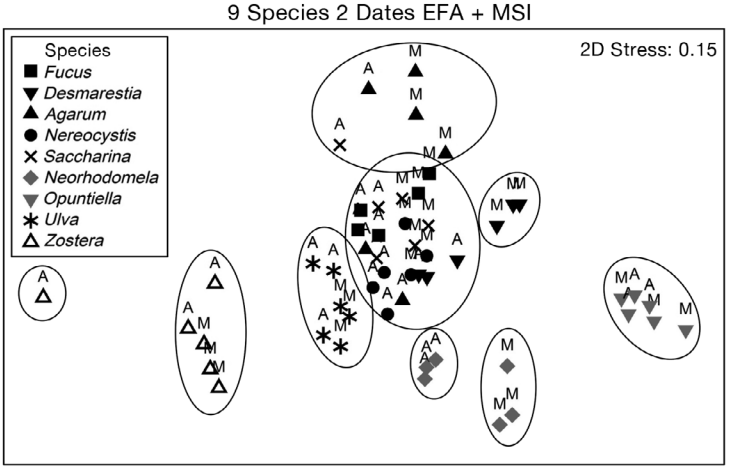

Fig. 6. MDS plot for essential fatty acids (EFAs) plus multiple stable isotopes (MSI) for 9 macrophyte species from Pt. Caution from May (M) and August (A) 2010. Each point is a replicate sample. Ellipses denote significantly different cluster groups (p = 0.05) identified in a SIMPROF analysis

similarity are clear regardless of date. For example, the 1 angiosperm (*Zostera*), 2 rhodophytes, 1 chlorophyte, and 5 ochrophytes are distinctly separated (Table 1, Fig. 6). Thus, while biomarkers found on one date would not necessarily allow us to discern the presence of a given species (e.g. one kelp versus another), they would allow us to denote the phylum present. Our species list was insufficiently broad to determine whether spatial or temporal patterns likewise are clear at a family or ordinal level (e.g. Galloway et al. 2012).

An additional temporal analysis using both EFAs and MSI for 3 kelps and *Zostera* over 3 dates (Table 1) distinguished 6 clusters (not illustrated); *Zostera* replicates from all dates were in 1 group characterized by low $\delta^{15}N$, low $\delta^{34}S$, and high 18:3ω3 (ALA). Each of the 3 kelp species from March was in its own group, all characterized by low $\delta^{15}N$ and high $\delta^{34}S$. *Agarum* from May and August were mostly distinct from the other kelps, distinguished largely by abundant 20:5ω3 (EPA). *Nereocystis* and *Saccharina* from May and August grouped largely together and were distinguished by abundant 18:4ω3 (SDA). Therefore, it is clear that even using all 9 EFA plus MSI variables, it is difficult to distinguish all species (especially the closely related kelps), and different dates have unique combinations of biomarkers.

Snurchin mixing model comparisons

In our first model simulation, as more variables (MSI and FAs) were added to the mixing model for diets of a theoretical consumer, the accuracy of diet prediction increased and the proportion attributed to food sources not in the model (% unknown) decreased (Fig. 7). The poorest results (only 70% similarity between predicted and actual) were found using only 3 stable isotopes, and the best results (97% similarity) when the 3 MSI and 6 EFA predictor variables were combined with 19 non-essential FA variables.

In the second model simulation that incorporated our observed spatial and temporal variation, accuracy of predictions of the Bayesian model varied from 63 to 97% (Table 2). The highest similarity to actual diets (Table 2) was seen in Scenario 1 in which the Snurchin diets integrated foods from only 1 site in 1 season, and the biomarkers from food sources entered into the model were from that specific site and season. The 2 least accurate scenarios were those where assumptions commonly

found for sampling regimes in the literature were violated (1) when food sources were sampled and averaged from only 1 season across all sites, while Snurchin was integrated from multiple seasons across all sites (Scenario 7); and (2) when food sources were sampled and averaged from 1 season across all sites, while Snurchin was sampled during (and integrated from) a completely different season (Scenario 8). In these last 2 scenarios, the models typically predicted a disproportionately large amount of the diet coming from *Opuntiella*, even in cases when the actual diet contained little to none of this food source.

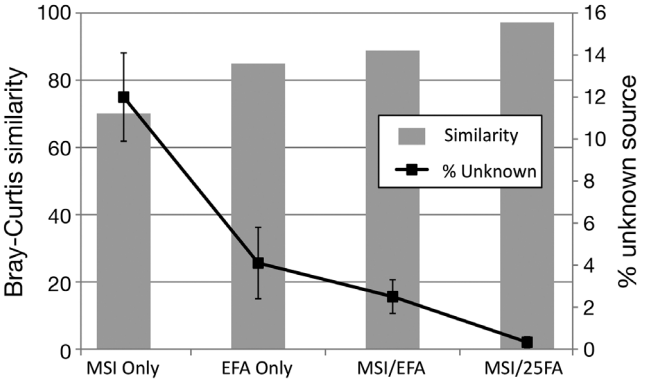

Fig. 7. Accuracy of mixing models for a theoretical consumer given 'diets' of species with known, variable biomarkers. Histograms show Bray-Curtis values for the similarities between each model prediction and the actual diet with given types of biomarkers, and the line shows percent of each diet attributed to unknown food sources

DISCUSSION

Our results call attention to the dangers inherent in the explicit and implicit assumptions in coastal food web studies of temporal and spatial stability of biomarkers. We found that both MSI and EFAs in marine macrophytes differ among sites and dates more than expected from most published literature. Both types of biomarkers are useful for distinguishing macrophyte taxa, although EFA signatures tended to be more consistent among replicate samples. If such data are used in mixing models to try to identify diets of consumers, the importance of the natural variation we observed depends strongly on the assumptions made about the space and time over which consumers integrate food sources.

MSI, especially for *Zostera marina*, varied strikingly between March (late winter) and May (spring), and less strongly between spring and late summer (August). $\delta^{13}C$ and $\delta^{15}N$ were particularly important in this change for all tested macrophytes, whereas $\delta^{34}S$ was less variable among dates except in *Zostera* (Fig. 3). We did not investigate the mechanisms responsible for these shifts; key seasonal changes could include isotopic concentrations of inorganic substrates in the water column, temperatures affecting kinetic reactions, or macrophyte growth rates affecting isotopic composition (Thompson & Calvert 1994, Fry 2006). Such temporal changes in MSI must be taken into account in biomarker studies (e.g. Hyndes et al. 2013); comparing values for primary producers in one month with consumers from another month, for example, could be very misleading.

The degree of spatial variation we found in MSI was somewhat surprising given the relative proximity (~30 km) of all of our sites. Even though the waters of the San Juan Archipelago are mixed by strong tides and currents, islands at the north and south ends apparently experience different enough conditions to result in comparatively local differences in isotopic composition. The nature of those differences was inconsistent, as seen in the strong site × species interaction terms (Table 3); for example $\delta^{13}C$ was relatively enriched at the northern site (Skipjack) for *Alaria*, but depleted for another kelp, *Saccharina*. Similar variability was seen in spatial patterns for $\delta^{15}N$ and $\delta^{34}S$, making it difficult to interpret the mix of factors influencing the fractionation processes. Salinity as well as freshwater inputs of N and C are all likely to change from north to south given the influence of the Fraser River; these may affect growth (and thus MSI values), but macrophyte species responded differently.

The use of biomarkers for tracking sources of primary production through aquatic food webs is increasingly common (e.g. Turner & Rooker 2006, Rooker et al. 2006, Budge et al. 2008, El-Sabaawi et al. 2010, Wing et al. 2012), but our understanding of potential limitations of these tools, especially for FAs, has lagged. While it is generally accepted that FA signatures of macrophytes will vary with environmental conditions (Floreto & Teshima 1998, Guschina & Harwood 2006, 2009, Becker et al. 2010), to our knowledge, researchers have not yet considered whether the seasonal variations seen in FA content (Honya et al. 1994, Nelson et al. 2002) are statistically or biologically relevant. Kelly & Scheibling (2012) recently cautioned that because of this uncertainty, researchers should document macroalgal biomarkers throughout the year. Our results demonstrate that even within a species, EFA can vary substantially among dates (Table 4). Moreover, this variation in

content may 'swamp out' the recently documented ordinal and family level resolution of FAs (Galloway et. al 2012) of the algal lineages. A key uncertainty of another kind is that relatively few studies have evaluated a consumer's ability to modify the FAs in its tissues from dietary FAs, for example in controlled feeding trials (but see Graeve et al. 1994, Hall et al. 2006, Kelly et al. 2008).

We found that stable isotopes and EFAs both discriminate among species (10 taxa) but FAs distinguished more species pairs than MSI, although it is difficult to evaluate how much this may be attributed simply to the greater number of biomarkers used in FAs. Crawley et al. (2009) similarly found that FAs provided 'more clarity' in both differentiating producers and determining the diets of consumers than did MSI. Different biomarkers work best at distinguishing different species—$\delta^{13}C$ for *Zostera*, $\delta^{34}S$ for *Opuntiella*, and certain FAs for certain kelps (see also Galloway et al. 2012). MSI are not useful for distinguishing among brown algae except for the atypical *Agarum*, which has substantially depleted $\delta^{13}C$ relative to other kelps (Fig. 3).

Our mixing model results from a hypothetical consumer given different 'diets' with biomarker variation on a scale seen in our field samples showed that the accuracy of the models increased with the number of predictor variables. In addition, the breadth of the spatial and temporal windows incorporated into the model greatly affected the results, and by extension, the assumptions going into the model are critical (see also Hyndes et al. 2013). The best results overall came from the scenario in which 1 season and 1 site were sampled for food sources and consumers, and the consumers were (correctly) assumed to only have integrated those specific food sources (Scenario 1, Table 2). However, when this assumption was violated by the consumers integrating food over multiple seasons (Scenario 7), or by sampling food sources in one season and consumers in another (Scenario 8), the model predictions of the consumer diet were very inaccurate. The literature often implicitly assumes that the temporal variation of food sources is not enough to significantly affect interpretation of MSI and FA signature analyses (Kharlamenko et al. 2001, Guest et al. 2008, Allan et al. 2010). While these sampling strategies and assumptions may often be necessary due to time and financial constraints, our data demonstrate that the resulting mixing model outcomes and the interpretations drawn from qualitative evaluations of biomarker patterns may lead to significantly different and potentially inaccurate conclusions about a consumer's diet.

The choice of biomarkers should depend on the question being asked. FAs appear to have a finer taxonomic resolution for macrophytes than MSI, clearly distinguishing family- and order-level differences (Galloway et al. 2012). Certain stable isotope ratios are useful in tracking gross environmental patterns that FAs cannot provide; for example, $\delta^{13}C$ tracks the photosynthetic pathway used to initially fix carbon, $\delta^{34}S$ tracks terrestrial versus marine sources, and $\delta^{15}N$ is frequently used to estimate the trophic length of a food web. A number of processes add variability to these isotopic values, providing a double-edged sword of uniqueness and uncertainty. Our simulations showed that combining many MSI and FA signatures increases accuracy of mixing models in predicting the contribution of primary producers to a simulated higher trophic level. Even in cases where temporal and spatial variation in biomarkers of primary producers overwhelms species-level resolution, phylum-level differences remain clear. Thus until researchers have demonstrated that site and seasonal variation does not confound interpretation of food web sources, it would be wise if inference about the contributions of primary producers to food webs was limited to phylum level. Additional work is needed on the sensitivity of mixing models to the levels of variation seen; our modeling with a hypothetical consumer suggests that assumptions about integration periods of food sources and tissue turnover rates are very important, but empirical data are needed.

By more rigorously examining the patterns in the variability of natural isotopes, food sources, and primary consumers, we should gain valuable insight into the scale, strength, and relative importance of varying fractionation and other processes affecting biomarkers. This in turn should result in a better understanding of food webs. A similar conclusion was reached by Woodland et al. (2012) about the need to identify 'dynamic baselines' for accurate isotopic characterization of freshwater primary producers. We therefore recommend that researchers incorporate sampling for biomarker variation into their study designs, and increase the number of biomarker variables used in mixing models; this will also enable increased resolution of multiple sources (Fry 2013).

In conclusion, for a diverse group of marine primary producers in our coastal case study, many of the biomarkers commonly used in trophic studies can vary significantly among species, sites within a region, and dates. Phylum-level variation is more constrained. FAs generally provide clearer separation of species than MSI, but using both types of bio-

markers together may mitigate the uncertainty caused by natural variation and thus may strengthen inferences. Because of this high spatial and temporal variation, mixing models run a substantial risk of drawing incorrect conclusions about actual sources of food if they assume that sources of organic matter in the diets of marine consumers are constant, or if they use values based on very restricted sampling. Further study is needed into how this variation propagates up food webs, e.g. the relative magnitude of change in biomarkers across dates and sites as compared with fractionation or modification upon incorporation into consumer tissues.

Acknowledgements. A. T. Lowe, S. Wiesner, K. Wilcox, and J. S. Yeung provided extensive technical and intellectual support. This work was funded by National Science Foundation (NSF) Grant OCE-0925718, the American Academy of Underwater Scientists (Kathy T. Johnston PhD support scholarship to A.W.E.G.), the International Phycological Society (Paul C. Silva Student Grant to A.W.E.G.), and an NSF OACIS-GK12 Teaching Fellowship DGE-0742559 (to A.W.E.G.).

LITERATURE CITED

Allan EL, Ambrose ST, Richoux NB, Froneman PW (2010) Determining spatial changes in the diet of nearshore suspension-feeders along the South African coastline: stable isotope and fatty acid signatures. Estuar Coast Shelf Sci 87:463–471

Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E Ltd, Plymouth

Becker S, Graeve M, Bischof K (2010) Photosynthesis and lipid composition of the Antarctic endemic rhodophyte *Palmaria decipiens*: effects of changing light and temperature levels. Polar Biol 33:945–955

Bell MV, Tocher DR (2009) Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions. In: Arts MT, Brett MT, Kainz M (eds) Lipids in aquatic ecosystems. Springer, New York, NY, p 211–236

Boecklen WJ, Yarnes CT, Cook BA, James AC (2011) On the use of stable isotopes in trophic ecology. Annu Rev Ecol Evol Syst 42:411–440

Bond AL, Diamond AW (2011) Recent Bayesian stable-isotope mixing models are highly sensitive to variation in discrimination factors. Ecol Appl 21:1017–1023

Brenchley JL, Raven JA, Johnston AM (1997) Resource acquisition in two intertidal fucoid seaweeds, *Fucus serratus* and *Himanthalia elongata*: seasonal variation and effects of reproductive development. Mar Biol 129: 367–375

Britton-Simmons K, Eckman JE, Duggins DO (2008) Effect of tidal currents and tidal stage on estimates of bed size in the kelp *Nereocystis luetkeana.* Mar Ecol Prog Ser 355:95–105

Britton-Simmons KH, Foley G, Okamoto D (2009) Spatial subsidy in the subtidal zone: utilization of drift algae by a deep subtidal sea urchin. Aquat Biol 5:233–243

Britton-Simmons KH, Rhoades AL, Pacunski RE, Galloway AWE and others (2012) Habitat and bathymetry influence the landscape-scale distribution and abundance of drift macrophytes and associated invertebrates. Limnol Oceanogr 57:176–184

Budge SM, Wooller MJ, Springer AM, Iverson SJ, McRoy CP, Divoky GJ (2008) Tracing carbon flow in an arctic marine food web using fatty acid-stable isotope analysis. Oecologia 157:117–129

Carvalho MC, Hayashizaki K, Ogawa H (2009) Carbon stable isotope discrimination: a possible growth index for the kelp *Undaria pinnatifida.* Mar Ecol Prog Ser 381: 71–82

Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial. PRIMER-E Ltd, Plymouth

Crawley KR, Hyndes GA, Vanderklift MA, Revill AT, Nichols PD (2009) Allochthonous brown algae are the primary food source for consumers in a temperate, coastal environment. Mar Ecol Prog Ser 376:33–44

Dalsgaard J, St. John M, Kattner G, Muller-Navarra DC, Hagen W (2003) Fatty acid trophic markers in the pelagic marine food environment. Adv Mar Biol 46:225–340

Eckman JE, Duggins DO, Siddon CE (2003) Current and wave dynamics in the shallow subtidal: implications to the ecology of understory and surface-canopy kelps. Mar Ecol Prog Ser 265:45–56

El-Sabaawi R, Sastri AR, Dower JF, Mazumder A (2010) Deciphering the seasonal cycle of copepod trophic dynamics in the Strait of Georgia, Canada, using stable isotopes and fatty acids. Estuaries Coasts 33:738–752

Fenton GE, Ritz DA (1989) Spatial variability of ^{13}C:^{12}C and D:H in *Ecklonia radiata* (C.Ag.) J. Agardh (Laminariales). Estuar Coast Shelf Sci 28:95–101

Finlay JC, Power ME, Cabana G (1999) Effects of water velocity on algal carbon isotope ratios: implications for river food web studies. Limnol Oceanogr 44:1198–1203

Floreto EAT, Teshima S (1998) The fatty acid composition of seaweeds exposed to different levels of light intensity and salinity. Bot Mar 41:467–481

Floreto EAT, Teshima S, Ishikawa M (1996) Effects of nitrogen and phosphorus on the growth and fatty acid composition of *Ulva pertusa* Kjellman (Chlorophyta). Bot Mar 39:69–74

Fry B (2006) Stable isotope ecology. Springer Science, New York, NY

Fry B (2013) Alternative approaches for solving underdetermined isotope mixing problems. Mar Ecol Prog Ser 472: 1–13

Galloway AWE, Britton-Simmons KH, Duggins DO, Gabrielson PW, Brett MT (2012) Fatty acid signatures differentiate marine macrophytes at ordinal and family ranks. J Phycol 48:956–965

Graeve M, Kattner G, Hagen W (1994) Diet induced changes in the fatty acid composition of arctic herbivorous copepods—experimental evidence of trophic markers. J Exp Mar Biol Ecol 182:97–110

Guest MA, Nichols PD, Frusher SD, Hirst AJ (2008) Evidence of abalone (*Haliotis rubra*) diet from combined fatty acid and stable isotope analyses. Mar Biol 153:579–588

Guest MA, Hirst AJ, Nichols PD, Frusher SD (2010) Multiscale spatial variation in stable isotope and fatty acid profiles amongst temperate reef species: implications for design and interpretation of trophic studies. Mar Ecol Prog Ser 410:25–41

Guschina IA, Harwood JL (2006) Lipids and lipid metabolism in eukaryotic algae. Prog Lipid Res 45:160–186

Guschina IA, Harwood JL (2009) Algal lipids and effect of environment on their biochemistry. In: Arts MT, Brett MT,

Kainz MJ (eds) Lipids in aquatic ecosystems. Springer, New York, NY, p 1–24

Hall D, Lee SY, Meziane T (2006) Fatty acids as trophic tracers in an experimental estuarine food chain: tracer transfer. J Exp Mar Biol Ecol 336:42–53

Hanson CE, Hyndes GA, Wang SF (2010) Differentiation of benthic marine primary producers using stable isotopes and fatty acids: implications to food web studies. Aquat Bot 93:114–122

Hemminga MA, Mateo MA (1996) Stable carbon isotopes in seagrasses: variability in ratios and use in ecological studies. Mar Ecol Prog Ser 140:285–298

Holst Hansen J, Berg Hedeholm R, Sünksen K, Tang Christensen J, Grønkjær P (2012) Spatial variability of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope ratios in an Arctic marine food web. Mar Ecol Prog Ser 467:47–59

Honya M, Kinoshita T, Ishikawa M, Mori H, Nisizawa K (1994) Seasonal variation in the lipid content of cultured *Laminaria japonica*: fatty acids, sterols, β-carotene and tocopherol. J Appl Phycol 6:25–29

Howe ER, Simenstad CA (2007) Restoration trajectories and food web linkages in San Francisco Bay's estuarine marshes: a manipulative translocation experiment. Mar Ecol Prog Ser 351:65–76

Howe ER, Simenstad CA (2011) Isotopic determination of food web origins in restoring and ancient estuarine wetlands of the San Francisco Bay and Delta. Estuaries Coasts 34:597–617

Hyndes GA, Hanson CE, Vanderklift MA (2013) The magnitude of spatial and temporal variation in $\delta^{15}N$ and $\delta^{13}C$ differs between taxonomic groups: implications for food web studies. Estuar Coast Shelf Sci 119:176–187

Ishikawa NF, Doi H, Finlay JC (2012) Global meta-analysis for controlling factors on stable carbon isotope ratios of lotic periphyton. Oecologia 170:541–549

Jackson AL, Inger R, Bearhop S, Parnell A (2009) Erroneous behaviour of MixSIR, a recently published Bayesian isotope mixing model: a discussion of Moore & Semmens (2008). Ecol Lett 12:E1–E5

Kelly JR, Scheibling RE (2012) Fatty acids as dietary tracers in benthic food webs. Mar Ecol Prog Ser 446:1–22

Kelly JR, Scheibling RE, Iverson SJ, Gagnon P (2008) Fatty acid profiles in the gonads of the sea urchin *Strongylocentrotus droebachiensis* on natural algal diets. Mar Ecol Prog Ser 373:1–9

Kharlamenko VI, Kiyashko SI, Imbs AB, Vyshkvartzev DI (2001) Identification of food sources of invertebrates from the seagrass *Zostera marina* community using carbon and sulfur stable isotope ratio and fatty acid analyses. Mar Ecol Prog Ser 220:103–117

McCutchan JH Jr, Lewis WM Jr, Kendall C, McGrath CC (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. Oikos 102:378–390

Mill AC, Pinnegar JK, Polunin NVC (2007) Explaining isotope trophic-step fractionation: why herbivorous fish are different. Funct Ecol 21:1137–1145

Nelson MM, Phleger CF, Nichols PD (2002) Seasonal lipid composition in macroalgae of the Northeastern Pacific Ocean. Bot Mar 45:58–65

O'Kelly CJ, Kurihara A, Shipley TC, Sherwood AR (2010) Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian Islands. J Phycol 46:728–735

Page HM, Reed DC, Brzezinski MA, Melack JM, Dugan JE (2008) Assessing the importance of land and marine sources of organic matter to kelp forest food webs. Mar Ecol Prog Ser 360:47–62

Parnell AC, Inger R, Bearhop S, Jackson AL (2010) Source partitioning using stable isotopes: coping with too much variation. PLoS ONE 5:e9672

Peterson BJ (1999) Stable isotopes as tracers of organic matter input and transfer in benthic food webs: a review. Acta Oecol 20:479–487

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Annu Rev Ecol Syst 18:293–320

Ramos P, González-Solís J (2012) Trace me if you can: the use of intrinsic biogeochemical markers in marine top predators. Front Ecol Environ 10:258–266

Rooker JR, Turner JP, Holt SA (2006) Trophic ecology of *Sargassum*-associated fishes in the Gulf of Mexico determined from stable isotopes and fatty acids. Mar Ecol Prog Ser 313:249–259

Schaal G, Riera P, Leroux C, Grall J (2010) A seasonal stable isotope survey of the food web associated to a peri-urban rocky shore. Mar Biol 157:283–294

Simenstad CA, Wissmar RC (1985) $\delta^{13}C$ evidence of the origins and fates of organic carbon in estuarine and nearshore food webs. Mar Ecol Prog Ser 22:141–152

Simenstad CA, Duggins DO, Quay PD (1993) High turnover of inorganic carbon in kelp habitats as a cause of $\delta^{13}C$ variability in marine food webs. Mar Biol 116:147–160

Stephenson RL, Tan FC, Mann KH (1984) Stable carbon isotope variability in marine macrophytes and its implications for food web studies. Mar Biol 81:223–230

Taipale SJ, Kainz MJ, Brett MT (2011) Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids in *Daphnia.* Oikos 120:1674–1682

Thompson PA, Calvert SE (1994) Carbon-isotope fractionation by a marine diatom: the influence of irradiance, daylength, pH, and nitrogen source. Limnol Oceanogr 39:1835–1844

Thomson RE (1994) Physical oceanography of the Strait of Georgia-Puget Sound-Juan de Fuca Strait system. Can Tech Rep Fish Aquat Sci 1948:36–100

Turner JP, Rooker JR (2006) Fatty acid composition of flora and fauna associated with *Sargassum* mats in the Gulf of Mexico. Mar Biol 149:1025–1036

Vinagre C, Salgado JP, Mendonça V, Cabral H, Costa MJ (2012) Isotopes reveal fluctuation in trophic levels of estuarine organisms, in space and time. J Sea Res 72:49–54

Wiencke C, Fischer G (1990) Growth and stable carbon isotope composition of cold-water macroalgae in relation to light and temperature. Mar Ecol Prog Ser 65:283–292

Wing SR, McLeod RJ, Leichter JJ, Frew RD, Lamare MD (2012) Sea ice microbial production supports Ross Sea benthic communities: influence of a small but stable subsidy. Ecology 93:314–323

Woodland RJ, Rodriquez MA, Magnan P, Glemet H, Cabana G (2012) Incorporating temporally dynamic baselines in isotopic mixing models. Ecology 93:131–144

Wyatt ASJ, Waite AM, Humphries S (2010) Variability in isotope discrimination factors in coral reef fishes: implications for diet and food web reconstruction. PLoS ONE 5:e13682

Yokoyama H, Tamaki A, Harada K, Shimoda K, Koyama K, Ishihi Y (2005) Variability of diet-tissue isotopic fractionation in estuarine macrobenthos. Mar Ecol Prog Ser 296: 115–128

Editorial responsibility: Just Cebrian, Dauphin Island, Alabama, USA

Submitted: September 11, 2012; Accepted: February 18, 2013
Proofs received from author(s): March 17, 2013