Isotopic evidence and consequences of the role of microbes in macroalgae detritus-based food webs

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ABSTRACT: Deep subtidal coastal food webs are increasingly a focus among coastal researchers, largely due to the reliance of these systems on subsidies of organic detritus donated from allochthonous sources. To better understand the dynamics of these food webs, researchers have frequently employed multiple stable isotope (MSI; $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) analysis to gain insights into the relative importance and origins of various sources of detritus. However, the role of microbial decomposition in these detritus-based food webs has been poorly quantified and frequently overlooked in MSI food web mixing models. In this study, we explicitly examined the ecological and MSI methodological effects of microbial decomposition of algal detritus. We found a relationship between $\delta^{15}N$ enrichment and microbial abundance on decomposing kelp blades, and evidence that this pattern is influenced by the underlying biochemistry of the kelp. C:N ratios supported the hypothesis that microbial biofilms utilize nitrogen from kelp detritus, rather than augment the detrital nitrogen content. The results also showed that microbial effects can introduce a non-negligible amount of error to MSI mixing models if left unquantified.

KEY WORDS: Detritus · $\delta^{15}N$ · Microbes · Isotopes · Kelp · Food webs · $\delta^{13}C$ · $\delta^{34}S$

INTRODUCTION

Deep subtidal coastal food webs are poorly understood, particularly in regards to the basal resources that support them. With few exceptions, most food webs ultimately rely on photosynthetic primary producers as the main sources of carbon and energy, and thus rely directly on sunlight. However, in atrophic ecosystems, such as the deep coastal subtidal zone, where there is no intrinsic primary productivity, the ultimate source of carbon within food webs is difficult to discern. Organisms there must rely on subsidies of organic matter from allochthonous sources (Polis et al. 1997), which arrive via detrital pathways from photic ecosystems. However, owing to the inaccessibility of deep subtidal ecosystems, the type of detrital material, its geographic origin, and its relative importance to aphotic food webs are impossible to observe directly. To better understand the role of potential detrital sources in these food webs, researchers often rely on biomarkers, such as multiple stable isotope (MSI) analysis.

A current debate centers largely on whether detrital subsidies are primarily derived from phytoplankton or marine macroalgae (Duggins et al. 1989, Kaehler et al. 2000, 2006, Nadon & Himmelman 2006, Kelly et al. 2012, Miller & Page 2012). Contrasting studies (as reviewed in Miller & Page 2012) have documented or rejected the idea that marine macroalgae play a critical role in deep subtidal food webs, or 'the kelp detritus hypothesis'. The role of the microbial loop in detrital pathways is frequently invoked in these debates, especially in regard to food quality and availability (Duggins & Eckman 1997, Norderhaug et al. 2003). However, with a few exceptions (Zieman et al. 1984, Caraco et al. 1998), there are very few quantitative data on the effects of microbial colonization on commonly used isotope biomarkers, despite the heavy reliance of the field on interpreting stable isotope data.

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Investigation of the microbial loop has demonstrated that it plays a key role in cycling of dissolved organic matter (DOM) and particulate organic matter (POM) (Fenchel 2008). Refractory material, such as dissolved organic carbon (DOC), is utilized by bacteria, which form aggregates large enough to be consumed by zooplankton and filter feeders, thus introducing previously inaccessible carbon into the food web (Fenchel 2008). Although recent studies have found extensive and specific microbial communities on kelp blades (Staufenberger et al. 2008, Bengtsson et al. 2010), little work has been conducted on the microbial utilization of large fragmentary algal detritus (Bengtsson et al. 2011).

Limited data support the idea that microbial communities may significantly affect the biogeochemistry of detrital kelp. Phlorotannins are a well-studied class of algal secondary metabolites that can act as deterrents to both herbivory and microbial colonization (Johnson & Mann 1986, Hay 1996, Targett & Arnold 1998, Van Alstyne et al. 1999, Nagayama 2002, Goecke et al. 2010, Dubois & Iken 2012). The C:N ratio is a common measure of food quality, where lower ratios (achieved either by removing carbon or adding nitrogen) reflect higher quality foods. Norderhaug et al. (2003) and Duggins & Eckman (1997) quantified these 2 common biochemical metrics in pulverized kelps during decomposition. Both found decreased phlorotannin levels in aged detrital kelp particulate matter, suggesting that secondary metabolites were rapidly lost during decomposition. Parallel decreased C:N ratios suggested that, once released from phlorotannin controls, microbes quickly colonized the blades and augmented the overall nitrogen content from outside sources. These findings have been used as evidence that aged algae are of higher nutritional value to consumers than fresh algae because of microbial activity. A study by Macko & Estep (1984) found that colonies of a marine microbe, *Vibrio harveyi*, displayed large isotopic fractionation rates when assimilating nutrients from the media they were grown in. Several studies also found isotopic differences between fresh and aged detrital algae (Macko et al. 1982, Hill & McQuaid 2009, Krumhansl & Scheibling 2012), suggesting the occurrence of microbial fractionation. Despite these findings, little work has been done to explicitly measure the effects of microbial decomposition on stable isotopes in detrital algae.

Studies investigating detritus-based food webs using MSI analysis frequently do not account for potential microbial alteration of food sources during decomposition (Decottignies et al. 2007, Page et al. 2008, Kelly et al. 2012). Methods in these studies and others are based instead on implicit assumptions that fresh source materials collected in the photic zone are isotopically similar to the detrital materials that are ultimately utilized by deep subtidal consumers. Given the extent of decomposition a fragment of detritus may experience en route from photic to aphotic ecosystems, these assumptions are questionable and may lead to misinterpretation of MSI data collected to address the kelp detritus hypothesis.

We therefore tested whether microbial colonization has any measurable effect on the biogeochemical composition of kelp detritus, including MSI (δ^{13}C, δ^{15}N, δ^{34}S), phlorotannin, and elemental content. We used MSI analysis to determine whether microbes represent a novel food source or an intermediate trophic level, followed by a mixing model simulation using a hypothetical consumer to assess the methodological consequences of microbial decomposition.

MATERIALS AND METHODS

Aging experiment

This study was conducted in July and August 2011 in the San Juan Islands of Washington State, USA. Blades of the prevalent shallow water macroalgae (kelps) *Saccharina subsimplex* (hereafter *Saccharina*) and *Agarum fimbriatum* (hereafter *Agarum*) were collected from <5 m depth on SCUBA, and cleaned of any visible epibionts by gentle scrubbing. These kelps are the top 2 contributors to benthic drift macroalgae within the San Juan Islands, with *Saccharina* comprising 37% and *Agarum* 11% of the total drift algal biomass available to benthic consumers (Britton-Simmons et al. 2009). These species also provide a comparison for phlorotannin concentrations, with *Agarum* having relatively high content (2 to 8% dry mass) compared with *Saccharina* (0.5 to 1.3% dry mass, as *Laminaria groenlandica*) (Van Alstyne et al. 1999).

In the laboratory, 4 blades of each species were allowed to decompose for 5 wk in complete darkness in a flow-through seawater system. This design encouraged the accumulation of natural microbial populations. Each blade was retained in a separate container so that they could not contaminate each other. Sample fragments (~5 × 5 cm) were dissected weekly from approximately the same location on each blade and frozen for later elemental, MSI, and phlorotannin analysis. Each sampled blade fragment was cut in two; one subsample was processed for analysis normally, while the other was scraped thor-

oughly with a razor blade and rinsed to remove any microbial biofilm that may have developed over the course of the experiment (Bengtsson et al. 2010). To test the efficacy of the microbe-reduction technique, additional samples were aged in tanks in complete darkness for 5 wk before freezing with the biofilms intact. Three subsamples were first processed for DAPI staining as described in 'Microbial abundances'. Subsamples from the same samples were then scraped thoroughly with a razor blade, rinsed in 0.22 µm filtered seawater, and placed in a new bag. The staining process was then repeated.

At the completion of the experiment, the accumulated material that had settled in the tanks from the flowing (unfiltered) seawater system was also collected for MSI analysis.

Kelp biogeochemistry

Experimental samples were processed for MSI (δ^{13}C, δ^{15}N, δ^{34}S) analysis using slightly modified methods from previous studies (Howe & Simenstad 2007, Page et al. 2008). Briefly, algal tissues were cleaned of epibionts via manual removal. The 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. The analyses were conducted at Washington State University's Stable Isotope Core laboratory using a DeltaPlus XP Isotope Ratio Mass Spectrometer, with a 2 σ analytical uncertainty of 0.5‰. Owing to cost constraints, only Week 1 and Week 5 samples were analyzed for *Agarum*, while we analyzed additional *Saccharina* samples from Week 3. Phlorotannin analyses on tissues from the same samples were conducted using a Folin assay, and expressed as percent composition of hydroxylated aromatic compounds (HAC) in wet kelp mass (Van Alstyne et al. 1999).

Microbial abundances

For both species, 3 blades were sampled at Week 1 and Week 5, and analyzed for microbial abundance via microscopy. All samples were first blotted and weighed, then vigorously palpated for 1 min in 10 ml of 0.22 µm filtered seawater. The effluent was immediately poured into a sterile 15 ml falcon tube, preserved with formalin at a concentration of ~2%, and then refrigerated for ~24 h before being plated onto slides. We diluted 0.5 ml of each effluent sample in

5 ml of 0.22 µm filtered seawater, added 10 µl of acridine orange to each, and quickly filtered each solution onto a 0.22 µm polycarbonate filter. Enough DAPI was added to each filter to cover the surface, and allowed to incubate for 10 min before being removed via filtration. The filters were then removed and mounted on slides. Counts were conducted using the field of view (FOV) method. The number of cells per FOV was counted for 20 FOVs, or until at least 1000 cells in total had been counted in very dense samples. An estimate of cells per gram of kelp wet mass was then calculated using the number of FOVs, the cells per FOV, dilution factors, and the blotted weight of the kelp sample. This method was also used to determine the standard deviation of each estimate. Owing to extremely patchy distribution in some samples, median values were used to evaluate microbial densities rather than mean values. This reduced the influence of outlier values to better approximate the true central tendency of the dataset.

Statistical analyses

We removed 1 *Saccharina* δ^{34}S data point as an outlier because it was more than 3 standard deviations from the mean. Owing to sampling error, only 3 replicates were obtained for elemental analysis of scraped samples of both species at Week 1 and Week 5. Week 3 *Agarum* samples were not analyzed due to cost constraints; as a result 'age' was analyzed as Week 1 versus Week 5 for both species to allow for the same statistical methods. Data were tested for normality using the Shapiro-Wilks test (Table 1). We analyzed normally distributed datasets with paired *t*-tests, using *a priori* selected contrasts. Non-normal datasets were analyzed using a Wilcoxon signed-ranks test and asymptotic significance is reported. Isotopic data and C:N ratios (Table 2) for each treatment group statistically analyzed were normally distributed (Table 1). Elemental percent composition data (Table 2) were not normally distributed (Table 1).

Elemental data for both species were compared as follows: unscraped Week 1 to Week 5; and scraped Week 1 to Week 5. Comparisons between scraped and unscraped samples were not conducted because non-C, -N, or -S material removed from the blades during scraping (e.g. NaCl, Si) could change the percent composition of the samples without changing the absolute elemental content; thus, only change over time was of interest for elemental data. Isotopic data for both species were compared as follows: Week 1 to Week 5 in unscraped samples; Week 1 to Week 5 in

Table 1. Results of Shapiro-Wilks test of normality for treated (scraped) and untreated (unscraped) *Agarum fimbriatum* and *Saccharina subsimplex* after 1 and 5 wk. Significance values of p < 0.05 (in **bold** with asterisks) indicate groups that have non-normal distributions

		Week	*Agarum* Statistic	df	p	*Saccharina* Statistic	df	p
C%	Unscraped	1	0.888	4	0.374	**0.667**	**4**	**0.005***
		5	0.852	4	0.234	0.933	4	0.611
	Scraped	1	0.919	3	0.447	0.983	3	0.747
		5	0.969	3	0.664	**0.754**	**3**	**0.009***
N%	Unscraped	1	**0.750**	**4**	**0.039***	0.904	4	0.452
		5	0.870	4	0.296	0.816	4	0.135
	Scraped	1	0.952	3	0.579	0.855	3	0.255
		5	**0.756**	**3**	**0.012***	0.909	3	0.414
S%	Unscraped	1	0.781	4	0.072	0.894	3	0.367
		5	0.959	4	0.775	0.871	4	0.302
	Scraped	1	0.926	3	0.475	0.988	3	0.791
		5	0.999	3	0.940	**0.750**	**3**	**0.000***
C:N	Unscraped	1	0.853	4	0.235	0.952	4	0.726
		5	0.978	4	0.891	0.837	4	0.186
	Scraped	1	0.822	3	0.169	0.891	3	0.359
		5	0.915	3	0.436	0.969	3	0.660
^{13}C	Unscraped	1	0.993	4	0.973	0.998	4	0.995
		5	0.993	4	0.973	0.907	4	0.465
	Scraped	1	0.981	4	0.906	0.950	4	0.718
		5	0.996	4	0.986	0.840	4	0.196
^{15}N	Unscraped	1	0.939	4	0.651	0.885	4	0.359
		5	0.811	4	0.124	0.929	4	0.588
	Scraped	1	0.993	4	0.972	0.910	4	0.483
		5	0.973	4	0.860	0.896	4	0.410
^{34}S	Unscraped	1	0.861	4	0.263	0.970	3	0.666
		5	0.800	4	0.103	0.887	4	0.368
	Scraped	1	0.939	4	0.651	0.939	4	0.651
		5	0.870	4	0.297	0.797	4	0.096

scraped samples; unscraped Week 1 to scraped Week 1 samples; and unscraped Week 1 to scraped Week 5 samples. We adjusted for multiple comparisons of MSI data using a Bonferroni correction to re-set α to 0.0125. Paired *t*-tests were conducted on HAC concentrations at Week 1 and Week 5 time points in both species. Differences in HAC concentration between species were evaluated using an independent *t*-test. Mean values were used to evaluate all datasets except microbial density. Microbial density comparisons were conducted using Kruskal-Wallis analysis; asymptotic significance is reported. All analyses were conducted using R (version 2.15) and SPSS (release 13.0) statistical software.

Mixing model

Simulated diets

We tested the sensitivity of a Bayesian mixing model to mild violations of the assumption that fresh algae are isotopically similar to aged algae by conducting a series of mixing model simulations. Our parameters were selected such that, for the purposes of the simulation only, 8 out of 9 macrophyte species used as diet sources did not change iso-

Table 2. Elemental composition and isotope values for treated (scraped) and untreated (unscraped) *Agarum fimbriatum* and *Saccharina subsimplex* after 1 and 5 wk

	C%	SD	n	N%	SD	n	S%	SD	n	C:N	SD	δ^{13}C	SD	n	δ^{15}N	SD	n	δ^{34}S	SD	n
Agarum																				
Unscraped																				
Week 1	29.05	2.03	4	2.39	0.13	4	0.99	0.19	4	12.17	1.22	−17.70	1.04	4	8.77	0.48	4	24.45	0.70	4
Week 5	22.41	3.07	4	1.83	0.20	4	1.09	0.15	4	12.27	1.23	−17.95	0.96	4	8.39	0.79	4	24.28	1.04	4
Scraped																				
Week 1	31.81	1.40	3	2.64	0.08	3	1.07	0.45	3	12.04	0.16	−18.11	0.69	4	8.84	0.49	4	23.06	0.87	4
Week 5	28.53	1.87	3	2.12	0.23	3	1.50	0.28	3	13.65	2.40	−17.84	0.88	4	8.52	1.07	4	22.32	0.75	4
Saccharina																				
Unscraped																				
Week 1	26.62	0.72	4	2.69	0.12	4	1.09	0.05	3	9.93	0.65	−14.32	1.34	4	6.79	0.78	4	24.36	0.25	3
Week 3	27.59	0.88	4	2.95	0.28	4	1.10	0.42	4	9.38	0.60	−13.64	1.69	4	7.63	0.31	4	24.13	0.97	4
Week 5	21.99	1.38	4	2.29	0.18	4	1.30	0.23	4	9.64	0.42	−14.08	2.15	4	8.23	0.62	4	22.56	0.43	4
Scraped																				
Week 1	30.66	1.27	3	3.12	0.08	3	1.49	0.42	3	9.82	0.23	−15.02	0.96	4	6.44	0.68	4	23.34	0.87	4
Week 3	28.49	0.67	4	2.77	0.21	4	1.04	0.37	4	10.31	0.83	−14.66	1.68	4	7.04	0.11	4	23.64	0.91	4
Week 5	28.04	0.91	3	2.43	0.18	3	1.54	0.19	3	11.58	0.50	−14.93	2.12	4	6.88	0.80	4	22.20	0.30	4
Deposited material																				
Week 5	3.62	0.11	3	0.55	0.03	3	0.59	0.07	3	6.63	0.16	−19.78	0.29	3	6.71	0.20	3	15.28	0.53	3

topically with age; in other words, they were isotopically identical whether they were fresh or aged. In this simulation, only *Saccharina* changed isotopically with increasing age. We generated 12 different diets for subpopulations of simulated consumers based on a subset of macrophyte MSI data from Dethier et al. (2013) combined with the kelp aging experiment (Tables S1 & S2 in the Supplement at www.int-res. com/articles/suppl/m494p107_supp.pdf). In this case, *Saccharina* was represented by Week 5 unscraped blades. A different macrophyte featured as the largest component (between 30 and 50%) in 10 of the diets, with contributions from each other algal species randomly assigned (between 0 and 30%) to add to 100%. An 11th diet distributed all contributions equally among macrophytes and a 12th diet was conducted in which aged *Saccharina* provided 100% of the consumers' diet.

Simulated consumers

The MSI signature for each individual 'consumer' was calculated as in Dethier et al. (2013). We used MSI data and contributions from the simulated diets described above to create a weighted average amalgam MSI signature for each diet. Amalgamated standard deviation was calculated for each diet based on the natural isotopic variation of each macrophyte species used, again weighted by diet contribution. Using the average amalgam MSI signature and standard deviation of each simulated diet, we generated 3 baseline amalgam signatures. The 3 amalgam signatures were then assigned a set of fractionation values for each predictor variable: C = 0.8‰ ± 0.09; N = 3.4‰ ± 0.10; S = 0.5‰ ± 0.31 (McCutchan et al. 2003, Yokoyama et al. 2005). By adding or subtracting natural variation in fractionation rates to mean fractionation rates (McCutchan et al. 2003, Yokoyama et al. 2005), we created a total of 9 different sample values for the 'consumer' for each of the 12 diet subpopulations to simulate realistic consumer variability. The MSI values of each consumer subpopulation were then entered into the Bayesian mixing model, Stable Isotope Analysis in R (SIAR; Parnell et al. 2010).

Model sources

To evaluate the effects on mixing model accuracy of a mild, aging-related violation of assumptions, the model sources were set up in 2 different ways: (1) a control for comparison (the model is given aged macrophyte isotope values as potential food input data, i.e. the exact diet of the simulated consumer); and (2) the standard assumptions were used (the model is given fresh macrophyte isotope values as potential food input data). 'Fresh' *Saccharina* was represented by Week 1 scraped blades, as 'fresh' samples are frequently scrubbed or scraped and then rinsed to remove epibionts (Guest et al. 2008, Page et al. 2008, Foley & Koch 2010). Fresh *Saccharina* was verified as isotopically distinct from all other sources before the analysis (Table S3 in the Supplement at www.int-res.com/articles/suppl/m494p107_supp.pdf). The diet composition predictions of the 2 scenarios were evaluated for accuracy using the Bray-Curtis similarity index on the compiled median predicted diets from the model versus the compiled 'actual' diets of the simulated consumer. The accuracy was re-evaluated on 2 subsets of the original diets: those in which consumer diets are more reflective of the actual bulk transport rates in the system (*Saccharina* provides ≥20% of diet), and those in which the consumers exert some preference (*Saccharina* is largest component of diet).

RESULTS

The validity of the manual scraping method used to reduce microbial abundances was verified by microscopy, with median microbial densities reduced by a range of 49 to 78%. While microbial populations were not eliminated completely, the reduction was deemed sufficient to proceed with the method.

Phlorotannin concentrations were species dependent but appeared to be unaffected by decomposition in either species (Fig. 1). Average phlorotannin concentrations expressed as percent wet mass HAC were not significantly different between Week 1 and Week 5 for either species. As a result, *Agarum* phlorotannin concentrations remained significantly higher than *Saccharina* (p = 0.005; Table 3, Fig. 1).

Table 3. Statistical analysis of phlorotannin concentration (expressed as hydroxylated aromatic compounds). Paired *t*-tests are between Week 1 and Week 5 time points in each species of kelp blades. Independent *t*-test is between both species at all time points

	t	df	p
Paired *t*-test			
Saccharina subsimplex	0.073	3	0.946
Agarum fimbriatum	1.183	3	0.322
Independent *t*-test			
Species	3.348	14	0.005

The sustained differences in phlorotannin concentrations were reflected in the microbial abundances; in *Saccharina*, median densities were 3.5 times higher in 5 wk old aged blades than in 1 wk old blades,

whereas in *Agarum*, microbial densities did not display strong trends with time (Fig. 2). Microbial distribution in *Agarum* samples was extremely clumpy, resulting in high standard deviations. Week 5 *Saccharina* samples had significantly higher microbial abundances than Week 5 *Agarum* samples (Kruskal-Wallis test, p = 0.05; Table 4), whereas there was no significant difference between Week 1 samples of both species (p = 0.83; Table 4).

Fig. 1. *Saccharina subsimplex* and *Agarum fimbriatum*. Average changes in phlorotannin concentration, expressed as percent hydroxylated aromatic compounds (HAC), over 5 wk of decomposition. Error bars are one standard deviation from the mean; values of each replicate are also plotted

Elemental compositions (expressed as percent of the sample) were different at an α level of 0.07 between 1 and 5 wk of decomposition for unscraped samples of both species. Given the small sample size of this experiment, such a p-value suggests a significant effect. Elemental carbon and nitrogen decreased in both species of unscraped kelps (p = 0.068 and p = 0.068 for *Agarum* carbon and nitrogen respectively; p = 0.068 and p = 0.068 for *Saccharina* carbon and nitrogen respectively; Table 5). Sulfur content increased over time in both species of unscraped kelp (Table 2) (p = 0.068 for *Agarum*, p = 0.285 for
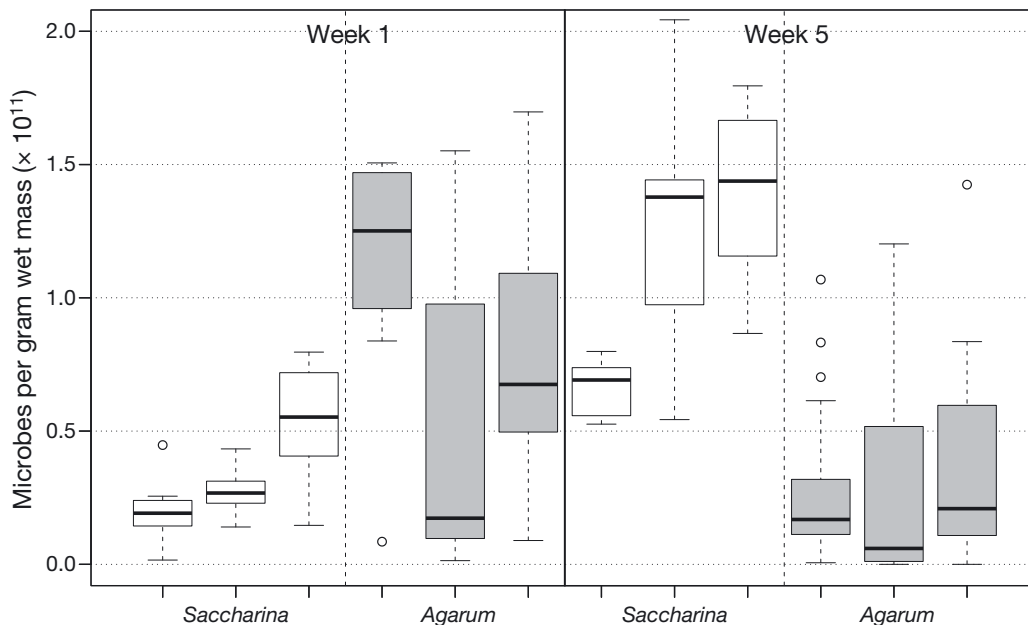

Fig. 2. *Saccharina subsimplex* and *Agarum fimbriatum* microbial abundance during decomposition. Each bar represents samples taken from a single blade. Week 5 samples were taken from the same blades as Week 1 samples, and appear in respective order (i.e. the 1st and 7th bar are from the same blade). Thick lines show median values; boxes show range of values within upper and lower quartiles; whiskers show range of values within 1.5 times the inter-quartile range

Saccharina; Table 5). In scraped samples of both species, there were no significant differences in elemental composition between 1 and 5 wk of decomposition (Table 5). Percent composition of each element was higher in scraped samples than in unscraped samples (Table 2), probably due to the removal of salt and sil-

ica that occurred during scraping, which would alter the apparent percent composition.

In unscraped samples of either species of kelp, the C:N ratio did not significantly change over the course of decomposition (p = 0.851 for *Agarum* and p = 0.493 for *Saccharina*; Table 6). However, the C:N ratio in scraped samples increased over time, though this was only significant in *Saccharina* (p = 0.382 for *Agarum*, and p = 0.023 for *Saccharina*; Table 6).

Table 4. Kruskal-Wallis analysis of microbial abundances between the 2 kelp species *Agarum fimbriatum* and *Saccharina subsimplex* at Week 1 and Week 5

Microbial abundance *Agarum* vs. *Saccharina*	χ^2	df	Significance
Week 1	0.05	1	0.83
Week 5	3.86	1	0.05

Table 5. Wilcoxon signed-ranks analysis of elemental percent composition data for *Agarum fimbriatum* and *Saccharina subsimplex* over time and treatment. Samples are named as follows: Un, unscraped blades; Sc, scraped blades; W1, Week 1 samples, and W5, Week 5 samples. Significance (Sig.) is 2-tailed

		Agarum		*Saccharina*	
		Z	Sig.	Z	Sig.
%C	Un W1 vs. Un W5	−1.826	0.068	−1.826	0.068
	Sc W1 vs. Sc W5	−1.604	0.109	−1.604	0.109
%N	Un W1 vs. Un W5	−1.826	0.068	−1.826	0.068
	Sc W1 vs. Sc W5	−1.604	0.109	−1.604	0.109
%S	Un W1 vs. Un W5	−1.826	0.068	−1.069	0.285
	Sc W1 vs. Sc W5	−1.604	0.109	0.000	1.000

Isotope composition

There were no significant differences in δ^{13}C, δ^{15}N, or δ^{34}S of *Agarum* blades in any of the pre-selected comparisons (Table 6). Week 5 blades that had been manually scraped were more deplete in δ^{34}S than Week 1 blades, although not at a significance level that met our Bonferroni corrected α of 0.0125 (p = 0.03; Table 6).

δ^{15}N in *Saccharina* blades was significantly different between Week 1 and Week 5 unscraped samples (p = 0.003, but was not significantly different between other pre-selected comparisons (Table 6). δ^{34}S of *Saccharina* blades was significantly more deplete in scraped Week 5 samples than in unscraped Week 1 samples (p = 0.001; Table 6), but differences between the other pre-selected comparison were not significant (Table 6). There were no significant differences in δ^{13}C in any of the pre-selected comparisons for *Saccharina* (Table 6).

There was an inverse relationship between the amount of elemental nitrogen in the *Saccharina* blade and δ^{15}N enrichment in both the scraped and unscraped kelp blades (Fig. 3). δ^{15}N of unscraped blades were enriched relative to the scraped kelp, and the amount of enrichment relative to the scraped kelp increased as elemental nitrogen decreased (Fig. 3). The intercept of the trendline for unscraped kelp samples was 12.13 (R^2 = 0.59), which provides an estimate for the isotopic value of a pure biofilm sample that has maximally fractionated its substrate. The intercept of the trendline for scraped kelp samples was 9.34 (R^2 = 0.28), which estimates kelp substrates that have been maximally fractionated. By subtracting the intercepts of both lines, the theoretical fractionation value (Δ)
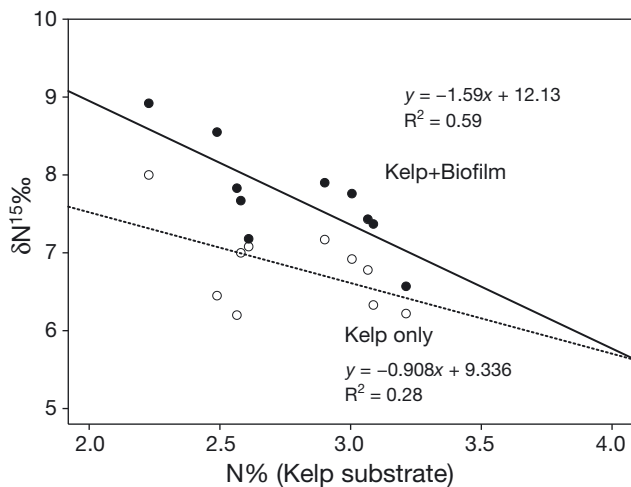
Table 6. Results of *a priori* selected paired *t*-tests for *Agarum fimbriatum* and *Saccharina subsimplex* over time and treatment. Samples are named as in Table 5. A Bonferroni correction was applied in the isotopic results; significance (Sig.) was noted only in contrasts where p < 0.0125 (asterisk, **bold**). In C:N results, no correction was applied. Significance (Sig.) is 2-tailed

		Agarum			*Saccharina*		
		t	df	Sig.	t	df	Sig.
δ^{13}C	Un W1 vs. Un W5	0.799	3	0.483	−0.234	3	0.830
	Sc W1 vs. Sc W5	−0.631	3	0.573	−0.341	3	0.755
	Un W1 vs. Sc W5	0.507	3	0.647	0.041	3	0.970
	Un W1 vs. Sc W1	1.222	3	0.309	0.456	3	0.679
δ^{15}N	**Un W1** vs. **Un W5**	0.618	3	0.580	**−9.227**	**3**	**0.003***
	Sc W1 vs. Sc W5	0.598	3	0.592	−1.395	3	0.257
	Un W1 vs. Sc W5	0.357	3	0.745	−0.192	3	0.860
	Un W1 vs. Sc W1	−0.324	3	0.767	4.627	3	0.019
δ^{34}S	Un W1 vs. Un W5	0.207	3	0.850	4.530	2	0.045
	Sc W1 vs. Sc W5	2.081	3	0.129	1.283	3	0.290
	Un W1 vs. **Sc W5**	3.912	3	0.030	**30.704**	**2**	**0.001***
	Un W vs. Sc W1	2.335	3	0.102	1.603	2	0.250
C:N	Un W1 vs. Un W5	−0.205	3	0.851	0.779	3	0.493
	Sc W1 vs. Sc W5	−1.111	2	0.382	**−6.515**	**2**	**0.023***

Fig. 3. Elemental nitrogen in scraped kelp versus δ^{15}N in scraped kelp (○) and kelp with biofilm (●)
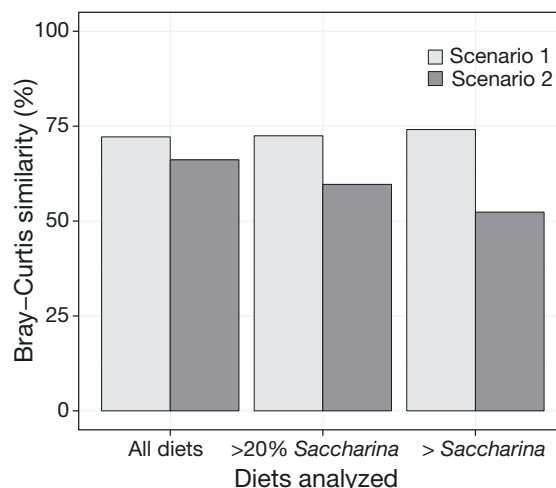

Fig. 4. Bray-Curtis similarity of predicted diets to actual diets under 2 modeling scenarios (Scenario 1 = microbially decomposed *Saccharina* used in source inputs, Scenario 2 = fresh *Saccharina* used in source inputs) and 3 diet subsets: (1) all diets considered; (2) diets composed of 20% or more *Saccharina*; and (3) diets in which *Saccharina* is the largest source

of a pure microbial biofilm relative to its substrate can be calculated to be 2.79‰ (SE = 0.6) at any point during decomposition. This value may be influenced by the presence of a single data point pulling the trendline of the scraped kelp samples upward (Fig. 3). Eliminating this single data point resulted in a trendline with an intercept of 7.29‰, and thus a substantial increase in the estimated fractionation value of the microbial biofilm. It is worth noting that by removing this single data point, R^2 drops to 0.02, suggesting no significant relationship between elemental nitrogen and ^{15}N in scraped blades. However, the data point in question did not meet our predefined criteria for outliers.

Deposited material

The MSI values of material deposited from the flowing seawater system over the course of the experiment bore little resemblance to *Saccharina* blades at any stage of decomposition (Table 2).

Mixing model simulations

When all diets for theoretical consumers were considered equally, the mixing model results were most accurate for the control, where aged algae were used as source inputs (Fig. 4, Scenario 1). In this instance, the Bray-Curtis similarity index of the median predicted diets versus the actual diets was 72.2%. This value was considered to be the maximum level of accuracy that could be achieved when using 3 bio-

markers to resolve the 9 macrophyte sources in our dataset. When standard methods of using only fresh algae that has been scrubbed to remove epibionts (analogous to Week 1 scraped in our data) as inputs were used (Scenario 2), the Bray-Curtis similarity index dropped to 66.1%. Differences among scenarios were more pronounced when only considering diets where *Saccharina* contributed 20% or more to the total actual diet (Fig. 4, Scenario 1 = 72.7%, Scenario 2 = 59.7%), and even more pronounced in diets where *Saccharina* was the largest contributor to the total actual diet (Fig. 4, Scenario 1 = 74.6%, Scenario 2 = 52.1%). Distributions of the model predictions for diets with *Saccharina* as the largest contributor are available in Figs. S1–S3A,B and Tables S4–S6 in the Supplement at www.int-res.com/articles/suppl/m494 p107_supp.pdf. The contribution of *Fucus distichus* was frequently overestimated when only fresh inputs were used and the actual contribution of *Saccharina* was moderately large (e.g. Fig. 5).

DISCUSSION

Our results indicate that microbial decomposition may significantly alter the biogeochemical composition of detrital kelp. Several of our findings represent a departure from the current understanding of algal decomposition, especially in regard to changes in nutritional quality; these may have far-reaching consequences both ecologically and methodologically.
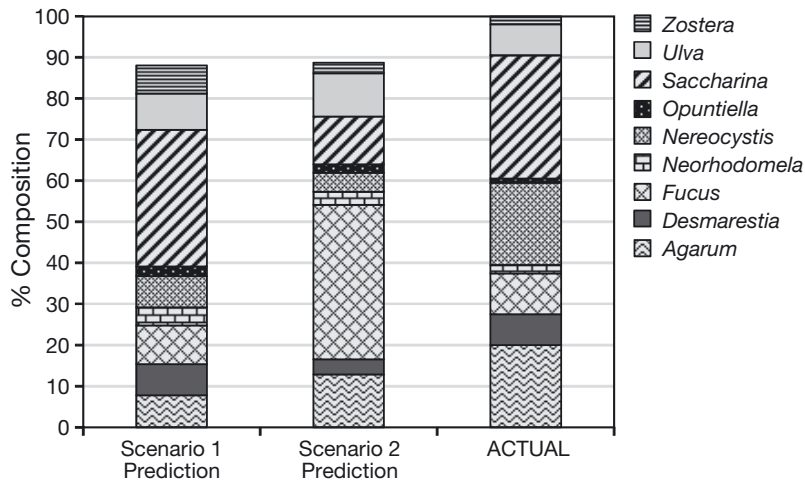
Fig. 5. Example of predictions for 1 of 12 diets under 2 modeling scenarios: Scenario 1 = microbially decomposed *Saccharina* used in source inputs; Scenario 2 = fresh *Saccharina* used in source inputs

Ecological consequences

Contrary to previous work (Duggins & Eckman 1997, Norderhaug et al. 2003), phlorotannin content did not decrease in either species of kelp over the course of decomposition. This difference is likely due to working with whole blades as opposed to pulverized particulate material, which may be more vulnerable to mechanical leaching of compounds due to an increased surface area. Our results do not necessarily suggest flaws in previous work; rather, whereas the results of Duggins & Eckman (1997) and Norderhaug et al. (2003) are applicable to kelp particles in POM, the present results are more applicable to larger kelp fragments.

Although the role of phlorotannins as an anti-herbivory defense has, at times, been questioned (Toth & Pavia 2002, Kubanek et al. 2004), phlorotannins are generally thought to deter both herbivory and microbial colonization (Johnson & Mann 1986, Hay 1996, Targett & Arnold 1998, Van Alstyne et al. 1999, Nagayama 2002, Goecke et al. 2010, Dubois & Iken 2012). The maintained phlorotannin levels in both kelps may therefore add a layer of complexity to the trophic status of macroalgal detritus. Detritus from macroalgal sources that contain high levels of phlorotannins, such as *Agarum*, may play a smaller role in detrital food webs (both as a direct food source and as a source of microbial productivity) than suggested by bulk transport data. Conversely, the role of algae with consistently lower phlorotannins may be amplified by a high rate of secondary productivity from microbial colonization, as seen in aged *Saccharina*, where concentrations may be too low to effec-

tively prevent microbial colonization. Alternatively, the differences in microbial abundance between the 2 species may be due to differences in the baseline C:N ratios of the kelps, but further research would be needed to explore this hypothesis (Findlay et al. 2002). Regardless, these findings suggest that the underlying algal biochemistry may be the main determinant of microbial dynamics on decomposing tissue.

Our results also indicated that aged algae are not of higher nutritional quality than fresh algae, at least in terms of the C:N ratio. C:N ratios did not drop over time as expected, and in fact the ratio increased over time in scraped samples for both kelp species. The apparent disagreement between this result and previous studies (Duggins & Eckman 1997, Norderhaug et al. 2003) may be due to an interaction between mechanical leaching of the kelp blades and microbial utilization. Carbon and nitrogen may be lost equally from the kelp blade during physical leaching of the substrate; however, due to enzymatic activity in the microbial biofilm, nitrogen (in the form of amino acids) may be absorbed into the biofilm preferentially over carbon (in the form of polysaccharides) (Smith et al. 1992, Martinez et al. 1996). Under these conditions, when the biofilm is retained as part of the kelp biocomplex, the majority of the net loss of C and N may be to the external environment in equal proportions, and the overall C:N ratio of the blade does not change. However, the results of our experiment demonstrate that if the biofilm is scraped from its kelp substrate, the nitrogen that was preferentially taken up by the biofilm from the kelp may be removed as well (Fig. 6a), resulting in an increased C:N ratio in the blade. Thus, rather than improving the nutritional quality of the food by increasing total nitrogen (where we would expect C:N to decrease in unscraped kelps, and remain the same in scraped kelps), as theorized in Duggins & Eckman (1997), the microbial biofilm may instead be acting as a sponge, absorbing organic molecules that would otherwise be lost to the environment.

While more research is needed to confirm this post hoc explanation and to investigate other possible factors (i.e. differing bacterial communities), this scenario resolves the apparent disparity in C:N ratios between our work with whole blades and previous work on pulverized blades representing kelp POM
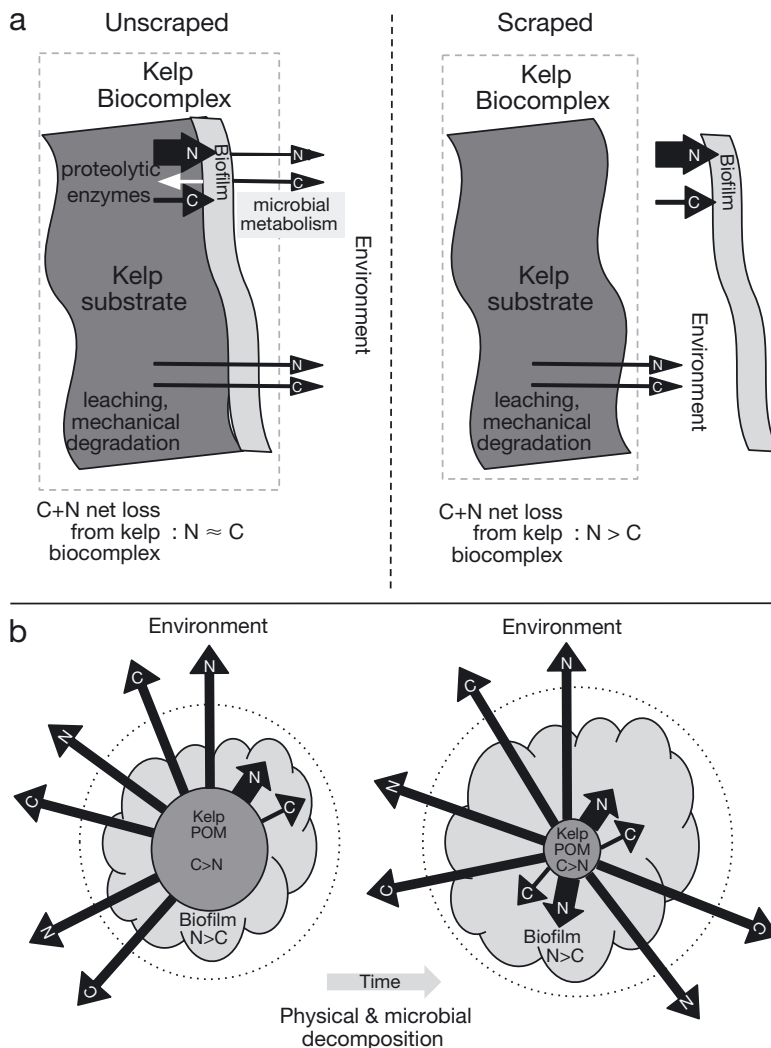
Fig. 6. Diagram of a proposed theory on the loss of elemental carbon and nitrogen from (a) large kelp detritus and (b) kelp particles. POM: particulate organic matter

(Duggins & Eckman 1997, Norderhaug et al. 2003). Increased surface area in pulverized blades may accelerate the physical leaching of carbon and nitrogen, to the point where there may be greater amounts of carbon and nitrogen in the microbial community that develops around each kelp particle than there is within the kelp particle itself (Fig. 6b). Thus, the preferential uptake of nitrogen over carbon in the biofilm becomes more apparent in POM.

Isotopic consequences

The underlying algal biochemistry may control which process dominates the decomposition of detri-

tal kelp — physical degradation or microbial digestion. These 2 pathways have very different impacts on the biogeochemistry of the detritus, and the kelp species used in this study provide insight into both. For instance, in *Agarum* detritus with or without biofilm, the lack of significant changes in MSI values during decomposition suggests that, in the absence of detritivores, mechanical degradation is the primary mode of breakdown, which is unlikely to exert MSI fractionation. This may be true in general of kelps with high phlorotannin levels or other antimicrobial defenses.

In contrast, the $\delta^{15}N$ isotopic shifts seen in the decomposition of *Saccharina* suggest a primary role of microbial activity. The results of our regression appear to show that the kelp substrate became increasingly enriched in ^{15}N as elemental nitrogen decreased, which could be explained as ^{14}N being preferentially removed due to the kinetic isotopic effects that occur during microbial enzymatic activity (Macko et al. 1986, Martinez et al. 1996). At the same time, the biofilm itself was enriched from the substrate by an estimated 2.7‰ (Fig. 3), which is within the range of empirically determined trophic enrichment factors in a number of marine organisms (Minagawa & Wada 1984). Simultaneously, the abundance of microbes in the biofilm increased by a factor of 3.5. These results suggest that the 'aged' *Saccharina* available to consumers in the deep subtidal is really a biocomplex of $\delta^{15}N$-enriched kelp substrate plus an even further $\delta^{15}N$-enriched biofilm.

Examination of our results suggests that $\delta^{34}S$ of unscraped *Saccharina* blades became depleted during decomposition, while no change was observed in *Agarum*; however, the effect in *Saccharina* was not statistically significant. This lack of significance was perhaps due to our low statistical power resulting from a small number of replicates; the difference was only significant when comparing Week 5 scraped with Week 1 unscraped samples. These results may suggest sulfur uptake in the microbial biofilm in *Saccharina*, but further research with greater statistical power would be needed to resolve this hypothesis.

Further evidence that the microbial colonization of detritus is regulated by underlying algal chemical defenses can be found in a concurrent study (Galloway et al. 2013), which analyzed a subset of samples from this experiment for fatty acid content. No effects of aging were found in the chemically defended *Agarum*. However, significant effects of aging on fatty acid content were found in less-defended *Saccharina* blades, accompanied by a trend of increasing bacterial-marker fatty acids.

An alternative explanation for the isotopic results of the present study might be that the apparent shifts are caused simply by POM settling out of the water column onto the kelp blades. If source mixing was occurring, the aged kelp MSI values would most likely shift towards that of the POM, reflecting the addition of new organic material. However, the MSI values of the accumulated material deposited from the seawater system were highly depleted relative to the kelps at any time point (Table 2), while $\delta^{13}C$ of the kelps did not change significantly over the course of decomposition and $\delta^{15}N$ of the kelps became enriched. Thus this pattern is more indicative of trophic fractionation than source mixing.

Applications to isotope-based research

The consequences of the different microbial fates of the 2 kelp species extend not only into the biogeochemistry of the kelp detritus biocomplex, but also into the interpretation of the structure of the food web above it when our understanding depends heavily on stable isotopes. The effect of the microbial biofilm behaving as a trophic level has the capacity to cascade up the food web. An individual of one species feeding on algal detritus may occupy a higher trophic level than another of the same species feeding on fresh algae. Likewise, an individual of one species in an area where the detrital algae pool is largely derived from chemically defended species of algae may occupy a lower trophic level than an individual of the same species in another area where the detrital algae pool is derived from algae with lower secondary metabolites. Two consumers feeding on the same piece of detrital algae may occupy different trophic levels depending on the feeding strategy of each, where surface grazers may utilize the biofilm to greater extent than shredders. On a much larger scale, in ecosystems where autochthonous primary productivity is low relative to microbial production based on allochthonous material, the entire community may shift upwards in trophic structure due to increased microbial activity (Galloway et al. 2013). Without taking microbial trophic levels into account, these shifts may be interpreted as evidence of consumers actively diet-switching to prey on other organisms, rather than passively ingesting microbes on detritus.

The isotopic shift that occurs during aging in some species of algae but not others adds a layer of complication when attempting to reconstruct a food web based on MSI mixing models alone. Microbial colonization during decomposition is likely controlled by the concentration of secondary metabolites in algal tissues; this is an area of research with room for exploration, as more studies investigate the many ecological roles of these metabolites (Engel et al. 2002, 2006, Goecke et al. 2010). Thus, depending on the biochemical profiles involved, some algal species may experience more microbial alteration of MSI values than others over the course of decomposition. Furthermore, there cannot be one single 'microbial' MSI signature, as different microbial assemblages may fractionate at different rates (Macko & Estep 1984). There is an increasing amount of research suggesting that each species of algae and even each part of an algal thallus can have a unique microbial assemblage (Stauffenberger et al. 2008, Bengtsson et al. 2010), and recent aging experiments on different algal species have shown variation in the isotopic changes that occur during decomposition (Hill & McQuaid 2009, Krumhansl & Scheibling 2012). Regardless, our results indicated that when investigating food webs in an ecosystem where detrital algae are readily available, using only fresh macrophytes as inputs in MSI mixing models may result in high prediction error in estimating the diet of a consumer. As an example, the subtidal kelp *Saccharina* was the largest dietary source in one 'actual' diet, contributing to 30% of the consumer diet, while the intertidal rockweed *Fucus* contributed only modestly at 10% (Fig. 5). Yet, a researcher relying on predictions based on fresh algae sources would erroneously conclude that *Fucus* was predominantly the dominant source in the consumer's diet. This inaccuracy is heightened as microbial effects occur in larger and larger proportions of diet sources (Fig. 4), yielding ecological interpretations that increasingly diverge from real-world happenings. Experimental determination of the isotopic shifts that occur during decomposition for at least the largest contributing algal sources (determined by gut content analyses or transport rates) is therefore ideal; any such shifts should be included when calculating macroalgal isotopic means and variances.

Additional uncertainty exists in the age of detritus. There is a clear gap in our knowledge about the retention time of kelp blades within detritus pools, as well as the length of time blades spend in transport to detritus pools. Previous work in Salt River Canyon in the US Virgin Islands estimated detrital seagrass and algal turnover to be on the order of days to months depending on environmental conditions (Josselyn et al. 1983), but this measure does not distinguish whether removal from the study system is due to transport, decomposition, or utilization. In our system, in the absence of herbivory, kelp blades senesce and dissipate after ~3 to 8 wk of decomposition (with *Agarum* taking the longest time, D. O. Duggins unpubl. obs.). A pool of detrital algae is probably a mixture of both species and ages. Incorporating the complex variability of composition, age, and microbial community on source algal species may prove to be a challenging but necessary next step in stable isotope ecology.

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