

## Using multiple tracers and directly accounting for trophic modification improves dietary mixing-model performance

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**Abstract.** Stable isotope (SI) mixing models are one of the most common approaches used to infer resource pathways in consumers. However, SI-based analyses are often underdetermined, and consumer SI fractionation is often unknown. The use of fatty acid (FA) biomarkers in mixing models offers an alternative approach that can resolve the underdetermined constraint. A limitation to both methods is the considerable uncertainty regarding the “trophic modification” of dietary tracers, which occurs when consumers transform dietary resources into their own tissues. We tested the utility of SI and FA approaches for inferring the diets of the marine benthic isopod (*Idotea wosnesenskii*) fed various marine macroalgae in controlled feeding trials. Our analyses quantified how the accuracy and precision of Bayesian mixing models were influenced by the choice of algorithm (SIAR vs. MixSIR), trophic modification (assumed or known), and whether the model was under or overdetermined (seven sources and two vs. 26 tracers) for cases where isopods were fed an exclusive diet of one of the seven macroalgae. Using the conventional approach (two SI with assumed trophic modification) resulted in average model outputs, that is, the contribution from the exclusive resource =  $0.20 \pm 0.23$  (0.00–0.79), mean  $\pm$  SD (95% credible interval), that only differed slightly from the prior assumption. This result was only somewhat improved by the use of measured trophic modification values for SI data. Using the FA-based approach with known trophic modification greatly improved model performance, that is, the contribution from the exclusive resource =  $0.91 \pm 0.10$  (0.58–0.99). The choice of algorithm (SIAR vs. MixSIR) made a clear difference when trophic modification was known, and the model was overdetermined. In this case, SIAR and MixSIR had outputs of  $0.86 \pm 0.11$  (0.48–0.96) and  $0.96 \pm 0.05$  (0.79–1.00), respectively. When using FA biomarkers, going from two to seven tracers dramatically improved model performance (i.e., from 0.55 to 0.89, respectively), whereas going from 13 to 26 tracers only improved performance by 0.01. This analysis shows the choice and number of dietary tracers and the trophic modification assumption greatly influence the performance of mixing-model dietary reconstructions and ultimately our understanding of what resources actually support aquatic consumers.

**Key words:** Bayesian mixing model; biomarkers; dietary tracers; fatty acids; feeding trials; herbivore; *Idotea wosnesenskii*; Special Feature: Biomarkers in Trophic Ecology; stable isotopes.

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### INTRODUCTION

Stable isotope (SI) mixing models have played an important role in aquatic and terrestrial

ecology, especially since the advent of the algorithms IsoSource, MixSIR, and SIAR greatly simplified and popularized this methodology (Phillips and Gregg 2003, Moore and Semmens

2008, Parnell et al. 2010). The most common application of these models is to infer the diets of consumers using analyses of the SI values of the consumers themselves and their prospective dietary resources. In most applications, two SI ratios (i.e.,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) are utilized, but some studies also employ a third tracer (usually  $\delta^{34}\text{S}$  or  $\delta^2\text{H}$ ; Fry 2006). Usually, more sources are considered than tracers are available to resolve these sources, causing these analyses to be mathematically underdetermined (Phillips and Gregg 2003, Boecklen et al. 2011, Fry 2013). There is currently a lack of clarity about the importance of this underdetermined constraint (Fry 2013, Semmens et al. 2013). Recent research suggests the mismatch between resources considered and SI tracers available may cause many Bayesian mixing-model analyses to be strongly biased toward the prior generalist assumption (Brett 2014, Galloway et al. 2015). Recognition of the importance of the underdetermined constraint calls for the implementation of additional dietary tracers in mixing-model analyses (Fry 2013, Phillips et al. 2014, Brett 2014, Galloway et al. 2015).

Fatty acids (FAs) hold considerable promise as dietary biomarkers in mixing-model applications because dietary FAs can leave strong signals in the lipid profiles of consumers (Dalsgaard et al. 2003, Iverson et al. 2004, Brett et al. 2006). Fatty acid profiles can also be highly diagnostic for particular basal resources (Dalsgaard et al. 2003, Brett et al. 2009, Galloway et al. 2012, Taipale et al. 2013). An important advantage with the use of FAs as basal tracers is that the lipid profiles of algal groups are primarily driven by phylogenetic relationships (Dalsgaard et al. 2003, Taipale et al. 2013). For example, while a myriad of environmental conditions affect algal FA composition (reviewed in Guschina and Harwood 2006), taxonomic affiliation explains three to four times more variation in phytoplankton FA profiles than do environmental conditions (Galloway and Winder 2015). Moreover, differences in macrophyte FAs in nature are less sensitive than SIs to seasonal and geographic variation (Dethier et al. 2013). An analysis of simulated zooplankton composed of “known” diets of three different phytoplankton resources showed that using 26 FA dietary tracers, compared to only using two SIs, resulted in much improved Bayesian mixing-model performance (Galloway et al. 2015).

One of the most important challenges to overcome in improving mixing-model analyses, regardless of the tracers used, is to properly characterize the trophic modification of the dietary biomarkers assimilated by the consumer. When consumers metabolize organic matter, the biomarker signals in their diets are modified prior to incorporation in their own tissues in a process that is often referred to as trophic fractionation in the SI literature and FA modification in the lipids literature. Here, we refer to this process for all biomarkers as “trophic modification” but still refer to marker-specific terms below. Many studies have identified the tendency for the heavier isotopes of carbon, and especially nitrogen, to become enriched in upper trophic-level consumers, and this fractionation is influenced by a wide range of factors including diet, consumer type, and the physiological state of the consumer (McCutchan et al. 2003). Despite this, many studies in the SI mixing-model literature simply assume the average SI fractionation derived from large-scale meta-analyses (e.g., Post 2002). Furthermore, the fractionation value assumed can have a profound influence on the outcomes of Bayesian models (Bond and Diamond 2011, Galloway et al. 2015). Similar to SIs, dietary FA profiles are modified in consumers. For example, Taipale et al. (2011) showed that *Daphnia* had less saturated FA and more highly unsaturated FA than their diets, and Strandberg et al. (2014) found that *Daphnia* retroconverted dietary 22:5 $\omega$ 6 to 20:4 $\omega$ 6. Fatty acid retention is also diet and consumer specific (Burns et al. 2011), so quantitative approaches for inferring consumer diet from their lipid profiles should always account for FA modification “natively” on the basis of detailed controlled feeding trials with defined diets (Iverson et al. 2004, Budge et al. 2012, Rosen and Tollit 2012, Galloway et al. 2014a, b, 2015).

The goal of this study was to directly compare the output accuracy and precision of SI and FA-based Bayesian mixing models for a real consumer and its known algal diets from the same experimentally generated biomarker data set. This analysis is based on a series of 10-week laboratory feeding trials where the marine isopod *Idotea wosnesenskii* was fed monospecific diets of seven different marine macroalgae from diverse phyla (i.e., Chlorophyta, Ochrophyta, and Rhodophyta) and families. The SI and FA

composition of the macroalgae and isopods fed these diets were determined. Because the isopods were grown from the neonate to the juvenile life stage, they grew to several times their initial size, and therefore, their final composition could be solely attributed to the monospecific macroalgal diets. We quantify how the performance of Bayesian mixing models is influenced by the type and number of dietary tracers used (i.e., two SI or 26 FA) and whether tracer trophic modification is directly determined or assumed. We also test the performance of the two most popular Bayesian algorithms (i.e., SIAR vs. MixSIR). Because our analysis is based on controlled feeding experiments, the true diets are known and mixing-model misclassification error can be easily quantified. We also tested whether isopod SI and FA trophic modification differed among the algal diets.

## METHODS

### Feeding trials

Ten-week feeding trials were conducted during the summers of 2012 and 2013 on fast growing juvenile *Idotea* using seven monospecific macroalgal diets (*Nereocystis luetkeana*, *Saccharina sessilis* [Laminariaceae]; *Fucus distichus* [Fucaceae]; *Ulva* sp. [Ulvacae]; *Mazzaella splendens* [Gigartiniaceae], *Porphyra* sp. [Bangiaceae], and *Smithora naiadum*

[Erythrotrichiaceae]). The algal diets were selected because (1) they are readily consumed by *Idotea*, (2) they represent the three major macroalgal phyla from six families, and (3) it has previously been shown that macroalgal taxa differ in their FA signatures at phylum and even family level ranks (Galloway et al. 2012). Experimental feeding trial methods were described in Galloway et al. (2014a). Briefly, *Idotea* neonates were removed from the brood pouches of gravid females and distributed randomly ( $n \approx 100$  per replicate) into triplicate 2-L aquaria per treatment diet (e.g., three experimental replicates; Fig. 1). Trials were conducted in a climate-controlled room ( $13.7^\circ \pm 1.4^\circ\text{C}$  [mean  $\pm$  SD], with a 16-hour light and eight-hour dark diel light cycle in aerated 0.3- $\mu\text{m}$  filtered seawater (changed every 48–72 h). Fresh algal material was provided with each water change to allow for ad libitum feeding. The isopods from every aquarium were measured at the start and end of the feeding trial (tip of the head to the end of the pleotelson). At end of the feeding trials, *Idotea* were starved for 24 h to purge their digestive tracts and their whole bodies were preserved for SI and FA analysis by freezing.

### Biomarker extraction and analysis

Samples were stored at  $-20^\circ\text{C}$  for <2 months, lyophilized for 48 h and ground to a fine powder prior to weighing and biomarker extraction.

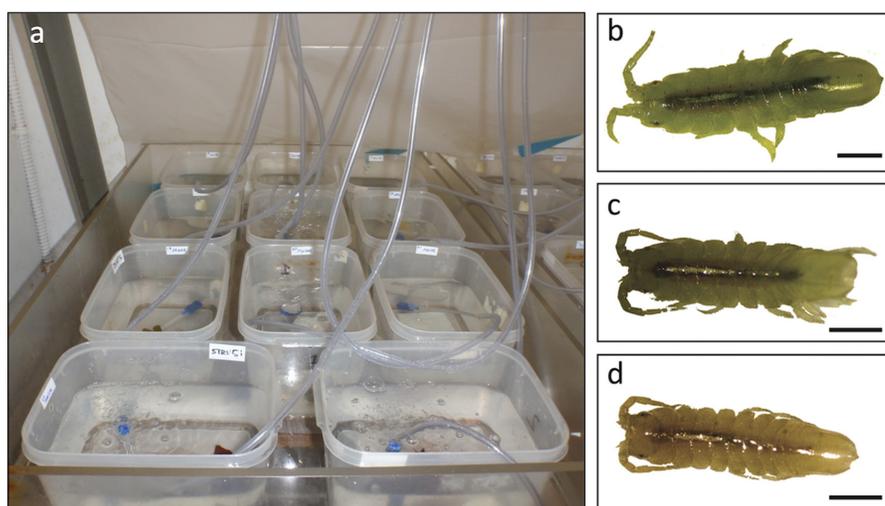


Fig. 1. (a) Photograph of the experimental array housing the isopod feeding trial, showing bubblers and 11 of the replicate 2-L aquaria. Representative photographs (with 1 mm scale bar) of *Idotea* fed (b) *Ulva* sp. (green), (c) *Nereocystis* (brown), and (d) *Mazzaella* (red) for 10 weeks (see Galloway et al. 2014a).

Dry, ground 10-mg aliquots of algal diets and isopods were retained for FA extraction and isotopic analyses. Multiple individual juvenile isopods were pooled into separate samples from each of the three replicate aquaria for each diet to achieve the required dry mass needed for analysis. The methodology for extraction and analysis of these particular FA samples is described in further detail in Galloway et al. (2014a). Isotope sample preparation and calculation of isotope ratios generally followed Howe and Simenstad (2007), except that replicate isopod samples were based upon whole-body homogenizations of ~10 juvenile isopods within each replicate aquarium. Stable isotope samples were not acid-washed or lipid-extracted because the lipid and the non-lipid tissues in the isopods are of critical interest to our study (see Matthews and Mazumder 2005). Stable isotope samples were weighed using a microbalance (2 mg) and enclosed in tin capsules for analysis at Washington State University's Stable Isotope Core Lab using a DeltaPlus XP Isotope Ratio Mass Spectrometer, with a  $2\sigma$  analytical uncertainty of 0.5‰.

#### *Mixing-model analyses*

We ran the Bayesian mixing-model analyses for the isotope feeding trial data for eight different combinations of code (SIAR or MixSIR), trophic modification (assumed or known), and tracer type (SI or FAs). To represent assumed trophic modification for SIs, we used the average values from Post (2002); that is,  $\Delta^{13}\text{C} = 0.4\text{‰} \pm 1.3\text{‰}$  and  $\Delta^{15}\text{N} = 3.4\text{‰} \pm 1.0\text{‰}$ . (Note that the convention used here is to represent isotope trophic fractionation within a consumer with  $\Delta\text{XY}$  and the isotope ratio of a sample with  $\delta^{\text{X}}\text{Y}$ .) To represent assumed trophic modification for FAs, we used the average difference for all treatments in the FA profiles of the macroalgal diets and the isopods that consumed these diets. To represent known trophic modification in the SI and FA analyses, we used the specific biomarker profiles of the isopods from each macroalgae treatment of our experiments. These consumer biomarker profiles were previously called the "consumer-resource library" by Galloway et al. (2015). We tested whether the mixing models correctly classified isopods fed each of the seven diets, where the outcome should have been 100% of the actual

resource used in the particular feeding trial and 0% for the other six resources. In each analysis, the FASTAR model returned the results for each potential diet item at the percentile level of resolution. From these data, we then calculated the output mean  $\pm$  SD, the median, and the 95% credible interval (i.e., the 2.5th to 97.5th percentile range of the model solution posterior density).

Because we compared different types and numbers of tracers in our analyses (i.e., two SI vs. 26 FA), the question of whether the type or number of tracers is most important cannot be directly answered. Therefore, we conducted an additional analysis to address this question. In this analysis, we compared the performance of the mixing-model when using two SI and two FA. We also tested all cases from two to 26 FA ( $n = 2, 3, 4, \dots, 26$ ). As there are millions of possible combinations of FA tracers that could have been tested, we ranked each FA tracer for inclusion or exclusion within the analysis based on its between-macroalgae treatment standard deviation, with tracers having the highest SDs selected first. This criterion selected FA tracers that were both highly variable between treatments and prevalent within the isopods. In all cases, we used known trophic modification and MixSIR.

#### *Statistical analyses*

We used boxplots to present biomarker trophic modification results (i.e., difference in biomarker values between diets and isopods fed those diets) and compared trophic modification for six tracers ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , palmitic acid [16:0], oleic acid [18:1 $\omega$ 9], arachidonic acid [20:4 $\omega$ 6; ARA], and eicosapentaenoic acid [20:5 $\omega$ 3; EPA]) among treatments using univariate ANOVA with Hochberg's GT2 post hoc tests (due to unequal sample size in treatments). The FAs included in this analysis are the four most abundant FAs across all samples. We also used ANOVA to quantify how the mean mixing-model outputs were affected by the choice of code, the trophic modification assumption, and tracer type for each of the seven diet scenarios tested. All univariate ANOVAs were calculated with SPSS v. 19.0 for Mac. We used non-metric multidimensional scaling (NMDS), calculated in the Vegan library in R (R Development Core Team 2014) and plotted with SPSS, as a multivariate visualization tool for the FA results.

## RESULTS

### *Biomarker trophic modification*

Trophic modification of FAs and SIs was variable among different algal diets (one-way ANOVAs for each tracer:  $\Delta^{13}\text{C}$   $F_6=11.72$ ,  $\Delta^{15}\text{N}$   $F_6=7.35$ , 16:0  $F_6=13.73$ , 18:1 $\omega$ 9,  $F_6=22.42$ , 20:4 $\omega$ 6,  $F_6=8.47$ , 20:5 $\omega$ 3,  $F_6=29.68$ ;  $P < 0.001$  for all tracers; Fig. 2). The mean  $\pm$  SD of  $\Delta^{13}\text{C}$  fractionation value ( $1.9\text{‰} \pm 3.0\text{‰}$ ; Fig. 2a) was higher than indicated by literature averages. Conversely, the average  $\Delta^{15}\text{N}$  fractionation value across all diets ( $1.6\text{‰} \pm 0.8\text{‰}$ ; Fig. 2b) was lower than generally indicated by the literature. In the case of  $\Delta^{13}\text{C}$ , the high mean and SD values were particularly influenced by the very high carbon fractionation values within the *Smithora* diet, that is,  $8.3\text{‰} \pm 0.8\text{‰}$ .

We compared the C:N ratios of the various diets and the isopods consuming these diets to the corresponding  $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$  values. The macroalgal molar C:N ratios were quite variable and ranged from a low of  $5.8 \pm 0.1$  for *Smithora* to a high of  $17.1 \pm 1.7$  for *Fucus*. The isopod C:N ratios were much less variable and ranged from a low of  $4.2 \pm 0.8$  for *Nereocystis* to a high of  $4.9 \pm 0.2$  for *Smithora*. There were no obvious statistical relationships between the macroalgal C:N ratios and the SI trophic modification values, except that *Smithora* had the lowest C:N ratios and isopods consuming *Smithora* had the highest  $\Delta^{13}\text{C}$  values. Across diets, macroalgal C:N ratios and isopod  $\Delta^{13}\text{C}$  values were not significantly correlated.

Trophic modification of the four most abundant FAs in the samples varied substantially among diets and was not consistent within phyla (Fig. 2c–f). For example, for the saturated FA 16:0, post hoc tests show that all brown algae and the green alga formed one group that differed from the red algae. However, species of red algae differed significantly from each other for 18:1 $\omega$ 9 and EPA, and species of brown algae differed from each other in ARA modification.

Trophic modification of algal diets by isopods is visualized in bivariate space for the two isotopes in Fig. 3a and for all 26 FAs in multivariate space using NMDS in Fig. 3b. The first axis of the NMDS was positively correlated ( $r = 0.94$ ) with EPA and negatively correlated with  $\alpha$ -linolenic acid (18:3 $\omega$ 3;  $\alpha$ -LA) and stearidonic acid (18:4 $\omega$ 3; SDA;  $r \approx -0.80$ ). The second axis was positively correlated ( $r = 0.94$ ) with ARA, positively correlated ( $r = 0.75$ ) with

18:1 $\omega$ 9, and negatively correlated ( $r = -0.75$ ) with the monounsaturated FA 18:1 $\omega$ 7. Without adjusting for trophic modification for any tracer, all isopods fell outside of the bivariate isotope resource polygon (Fig. 3a) and within the multivariate FAs resource polygon (Fig. 3b). Applying a conventional isotope trophic correction (e.g., values from Post 2002) did not cause isopods to fall within the bivariate resource polygon (Fig. 4b).

### *Mixing-model analyses*

The ANOVA showed the mean model outputs were strongly dependent on whether fractionation was assumed or known and the type of tracer used for the analyses, as well as the interaction term for these two factors (Table 1). The mixing-model mean outputs were much closer to the true value (i.e., 100%) when both the trophic modification was known and when FAs were used as a tracer (Table 2). The code used for these analyses also had a significant effect on the outputs, with MixSIR giving more accurate results.

In the most common case for Bayesian mixing models (where two SIs are used as the dietary tracer and isotope fractionation is assumed), the model outputs were barely differentiated from the model prior assumption (i.e., that each of the seven resources is equally important or  $\approx 14\%$ ). When the SIAR code was used, the model outputs for the true diet averaged slightly above the prior, that is,  $0.16 \pm 0.14$  (0.00–0.52), mean  $\pm$  SD (95% credible interval). When MixSIR was used, the mixing models performed worse than the prior assumption, that is, mean values  $\approx 0.03$  to 0.11, in five cases, and performed well (i.e., mean values  $\approx 0.63$ –0.70) in two cases (Table 2).

When the known SI fractionation values were used with two tracers (Fig. 5a), model performance improved only slightly for SIAR, that is, the mean output was  $0.18 \pm 0.16$  (0.00–0.58). When MixSIR was used, the outputs were slightly better than the prior in three cases, that is, mean  $\approx 0.18$ –0.19, somewhat better than the prior in one case (i.e., mean = 0.29), and much better in three cases (i.e., mean  $\approx 0.64$ –0.92; Fig. 5a).

When FAs were used as the tracers and general trophic modification values were assumed, mixing-model performance varied considerably. When SIAR was used, the mixing models performed below the prior in two cases (i.e., mean  $\approx 0.08$ ), at the prior in one case, better than the prior (i.e.,

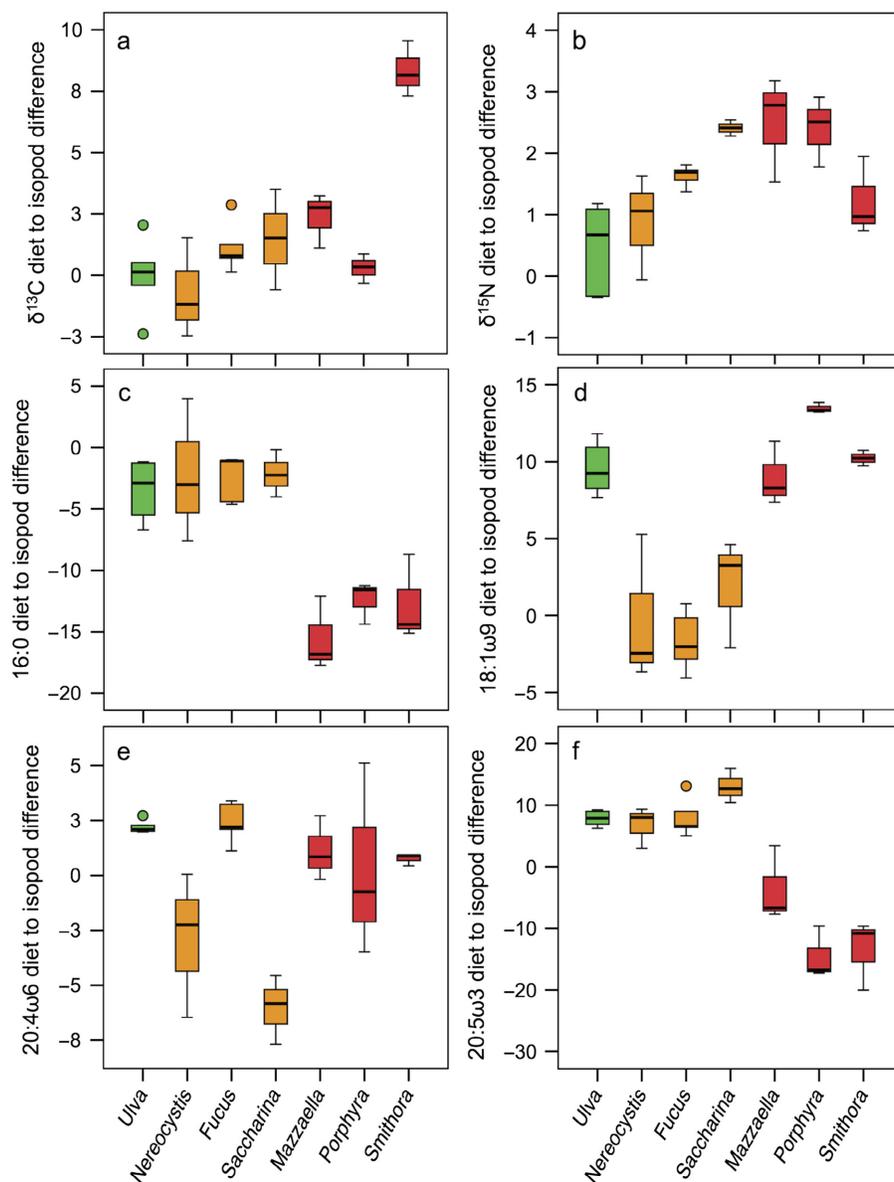


Fig. 2. Boxplots of measured isopod tracer trophic modification, which is the difference between tracer value of the algal diet (x-axis) and isopods fed those diets. Panels are a subset of individual tracers: (a)  $\delta^{13}\text{C}$ , (b)  $\delta^{15}\text{N}$ , (c) 16:0, (d) 18:1 $\omega$ 9, (e) 20:4 $\omega$ 6, and (f) 20:5 $\omega$ 3. The fatty acids shown are the four most abundant fatty acids in the samples. Boxplots show the median, quartile range, and 95% CI whiskers and outlier points.

mean  $\approx$  0.30–0.43) in two cases, and much above the prior (i.e., mean  $\approx$  0.65–0.68) in two cases. When MixSIR was used, the outputs tended to fixate on one or two resources, and usually the wrong ones. With MixSIR and assumed trophic modification with FA data, the results were extremely poor in three cases (i.e., mean  $\approx$  0.00–0.02), at the prior in one case, slightly above the prior on one case (i.e.,

mean = 0.23), and much above the prior in two cases (i.e., mean  $\approx$  0.59–0.77).

The Bayesian mixing models performed far better when known trophic modification values and FA data were used (Fig. 5b). In this case, SIAR had an average output of  $0.86 \pm 0.11$  (0.48–0.96) and MixSIR had an average output of  $0.96 \pm 0.05$  (0.79–1.00).

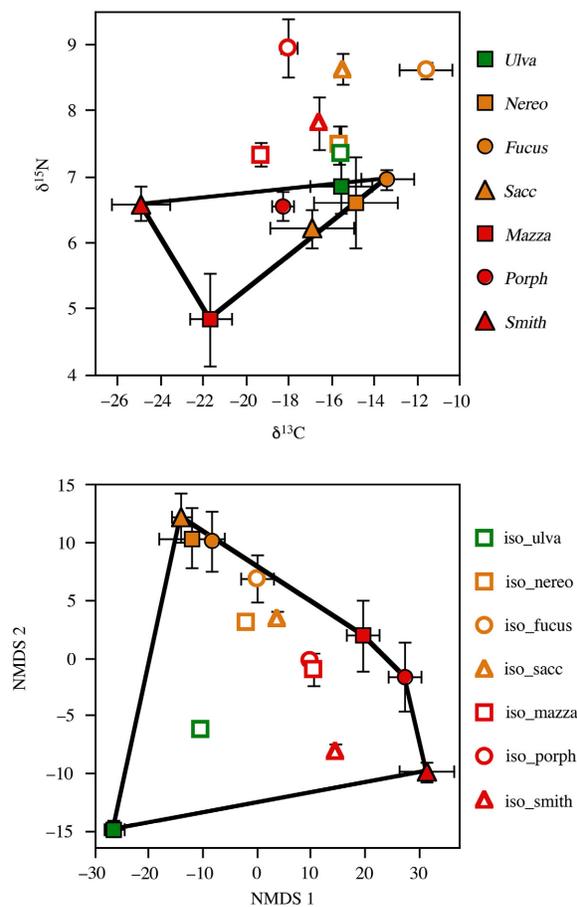


Fig. 3. Bivariate resource polygons based on the stable isotope data (upper panel) and the ordinates from a non-metric multidimensional scaling (NMDS) based on the macroalgae and isopod fatty acid data (lower panel). The polygons are defined by the most outer mean values of the algal diets. The error bars represent  $\pm 1$  SD; in most cases, for the isopod fatty acid data, the error bars are smaller than the plot symbols. The polygons depicted in these plots describe the outer boundaries for the resources used in our analyses. The isopod data in these panels have not been corrected for trophic modification. Closed symbols are algal diets and open symbols are isopods fed those corresponding diets. Colors of symbols correspond to different algal phyla (orange: Ochrophyta; red: Rhodophyta, green: Chlorophyta).

#### Type or number of tracers?

When we used two SI tracers, the average model outcome was  $0.48 \pm 0.31$  (0.01–0.92), and this improved to  $0.55 \pm 0.35$  (0.01–0.98) when two FA tracers were used. When additional FA

tracers were included in the analysis, model accuracy improved dramatically, but then leveled off when a large number of tracers was used (Fig. 6). For example, model performance improved dramatically, compared to only using two tracers, when seven FA tracers were used, that is,  $0.89 \pm 0.12$  (0.54–0.99). Model performance increased somewhat more when going from seven to 13 tracers, that is,  $0.943 \pm 0.07$  (0.73–1.00). Finally, model performance only improved slightly when going from 13 to 26 tracers, that is,  $0.955 \pm 0.05$  (0.80–1.00; Fig. 6).

## DISCUSSION

Our results showed that when attempting to resolve an underdetermined resource polygon with the conventional approach (i.e., two SI tracers and assumed fractionation), Bayesian mixing models gave outcomes that were only slightly better than the prior generalist assumption (i.e., an equal contribution to the consumer from all sources). When FA tracers were used and lipid trophic modification was known, the Bayesian mixing models gave very accurate and precise results, especially when MixSIR was used. Going from two to seven FA biomarkers dramatically improved model performance. Because our analyses included seven resources, this outcome suggests it is advantageous to have a similar number of tracers and resources in dietary mixing models. Conversely, going from 13 to 26 FAs only improved model performance slightly. These results show that if properly calibrated, FA-based Bayesian mixing models can give excellent results even for relatively complex resource polygons (e.g., Fig. 3b).

Within the ecosystems research community, there is great interest in which basal resources support upper trophic-level production. In the last decade, Bayesian mixing models utilizing two or three SI dietary tracers have overwhelmingly become the most common approach to analyze this class of questions. Several authors have suggested that in underdetermined cases (i.e., when the number of resources assessed is larger than the number of tracers used by two or more), these models may yield erroneous outcomes (Boecklen et al. 2011, Fry 2013). The underdetermined algebraic constraint for these types of problems is well known (Phillips and Gregg 2003), although some authors have claimed

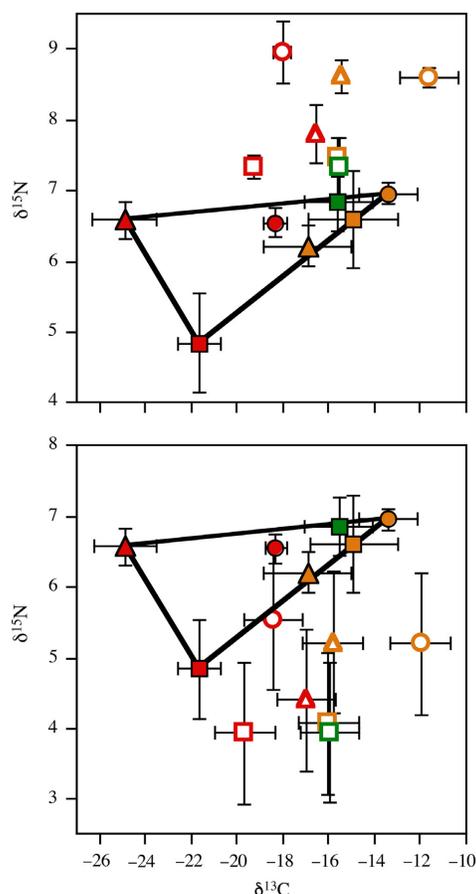


Fig. 4. The previously depicted uncorrected macroalgae and isopod stable isotope values (upper panel) and conventionally corrected (Post 2002) isopod stable isotope values (lower panel). The isopod error bars in the lower panel represent the uncertainty for stable isotope trophic modification. The symbols, polygons, and color descriptions follow Fig. 3.

Bayesian mixing models are robust to violations of their core assumptions and work exceptionally well even in underdetermined systems (Parnell et al. 2010). However, it is unclear how Bayesian mixing models can alleviate this fundamental algebraic constraint. The evidence Parnell et al. (2010) presented to support this assertion only demonstrated that the correct answer fell within the 95% credible interval for the model outputs for a hypothetical resource polygon with very low uncertainty. More recently, Brett (2014) showed the outputs of Bayesian mixing models are extremely sensitive to both uncertainty

in the SI ratios of the resources considered and violations of the underdetermined constraint.

#### *The importance of tracer type and number*

Our analyses showed the number of tracers used was much more important for model accuracy than the type of tracer used. The mixing model performed somewhat better when two FA tracers were used compared to using two SI tracers. However, in this case, we compared the two most informative FA tracers to the only two SI tracers that we had available. Had we tested all possible combinations of two FA tracers ( $n = 325$ ), it is likely that the SI tracers would have performed equally or better than the FA biomarkers. When going from two to seven FA tracers, there was a dramatic improvement in accuracy as well as a major reduction in the 95% credibility interval. Conversely, when going from 13 to 26 tracers, the accuracy and credibility interval only improved slightly. This comparison demonstrates that some FA biomarkers were much more informative than others. However, since FA analyses typically generate data for 20–30 molecules, it is also important to note that these results show there is no detriment to model performance when all available FAs are included in the analysis.

#### *Nitrogen trophic enrichment*

Our results are consistent with those from previous studies (Bunn et al. 2013) showing nitrogen fractionation can be quite different from the global mean value that many studies assume,

Table 1. ANOVA results for the means of the seven diets that should have classified as 100% for the eight cases that are described in this table.

Factor	df	SS	F	P	Var. expl.
Algorithm (A)	1	0.159	4.56	0.0379	0.025
Fractionation (B)	1	1.916	54.85	0.0001	0.305
AB	1	0.094	2.69	0.1075	0.015
Determined (C)	1	1.649	47.22	0.0001	0.263
AC	1	0.061	1.75	0.1925	0.010
BC	1	0.715	20.47	0.0001	0.114
ABC	1	0.009	0.26	0.6128	0.001
Error	48	1.676			0.267

Notes: df represents degrees of freedom, SS represents sum of squares, and var. expl. stands for the proportion of the SS explained by a particular factor. This variable is analogous to a conventional  $r^2$ , that is, model SS/total SS.

Table 2. The average outcomes for the eight cases tested for this analysis.

Case	Tracers	Fractionation	Code	Mean	SD	Median	2.5th percentile	97.5th percentile
1	Stable isotopes	Assumed	SIAR	0.159	0.144	0.119	0.004	0.518
2	Stable isotopes	Assumed	MixSIR	0.224	0.281	0.069	0.001	0.811
3	Stable isotopes	Known	SIAR	0.196	0.167	0.152	0.005	0.592
4	Stable isotopes	Known	MixSIR	0.476	0.307	0.498	0.012	0.923
5	Fatty acids	Assumed	SIAR	0.317	0.246	0.270	0.008	0.771
6	Fatty acids	Assumed	MixSIR	0.301	0.263	0.282	0.000	0.814
7	Fatty acids	Known	SIAR	0.856	0.114	0.888	0.480	0.963
8	Fatty acids	Known	MixSIR	0.953	0.054	0.971	0.795	0.995

*Note:* These results represent the aggregated outputs for the seven feeding experiments where the isopods were fed mono-specific macroalgal diets, for example, the outputs for *Ulva* when the isopods exclusively consumed *Ulva*.

that is,  $\Delta^{15}\text{N} = 3.4\text{‰} \pm 1.0\text{‰}$  (Post 2002). The data from our study indicated an average  $\Delta^{15}\text{N}$  fractionation value of  $1.6\text{‰} \pm 0.8\text{‰}$  while Bunn et al. (2013) reported an average of  $1.2\text{‰} \pm 1.3\text{‰}$ . The average  $\Delta^{15}\text{N}$  fractionation value we obtained was only half as large as the conventional  $3.4\text{‰}$  value and would therefore also imply a quite different trophic level in the isopods. The deviation between the  $\Delta^{15}\text{N}$  values we quantified in our laboratory experiments and the commonly assumed value is particularly problematic because the macroalgal resources considered in our analysis had small differences in their  $\delta^{15}\text{N}$  values, with minimum and maximum values of  $4.8\text{‰}$  and  $7.0\text{‰}$ , respectively, and a standard deviation for all  $\delta^{15}\text{N}$  values of only  $0.7\text{‰}$ . When the isopods were not fractionation-corrected, they all fell above the resource-mixing polygon. Conversely, when the conventional trophic modification factor (Post 2002) was applied, the consumers always fell below the resource polygon (Fig. 4).

#### Compound-specific stable isotope analyses

The results of this study have important implications for compound-specific analyses in food web ecology. Currently, compound-specific methods can be used to obtain much more accurate basal resource  $\delta^{13}\text{C}$  values by determining the SI ratios of FAs that are characteristic of particular algal groups (Taipale et al. 2015). Compound-specific amino acid (AA)  $\delta^{15}\text{N}$  analyses can also be used to more precisely characterize the trophic positions of consumers (McClelland and Montoya 2002, Nielsen et al. 2015). McMahan et al. (2015, 2016) have also used analyses of the  $\delta^{13}\text{C}$  values of essential AAs to

infer trophic pathways from basal resources to corals and fish using Bayesian mixing models. These studies usually employ five essential AA tracers, which is a substantial improvement over bulk SI analyses where usually two, and sometimes three, tracers are used. This method is based on the assumption that consumers incorporate essential AAs directly from their diets with minimal trophic modification and therefore signatures propagate through food webs unmodified. Future research should characterize the variability in the trophic modification of essential AA  $\delta^{13}\text{C}$  values of more consumers across a broad range of conditions (Nielsen 2016). Larsen et al. (2013) showed it is possible to use 10 or more AA biomarkers to differentiate between diverse basal resources (e.g., fungi, heterotrophic bacteria, phytoplankton, macroalgae, sea grasses, and terrestrial vegetation). Combining AA and FA biomarkers within a single mixing-model application is particularly promising because AAs represent protein trophic transfer and FAs represent lipid trophic transfer, so melding the two approaches could provide a much more nuanced perspective on food web processes.

#### Resource polygon geometry

Brett (2014) showed the geometric characteristics of SI-based resource-mixing polygons have a dramatic influence on the accuracy of Bayesian mixing models. Brett (2014) only considered idealized polygons where the resources considered were always equispaced and defined the external boundaries of the polygons, that is, equilateral triangles, squares, etc. In the present analysis, several of the resources considered were closely clustered and in the interior of the

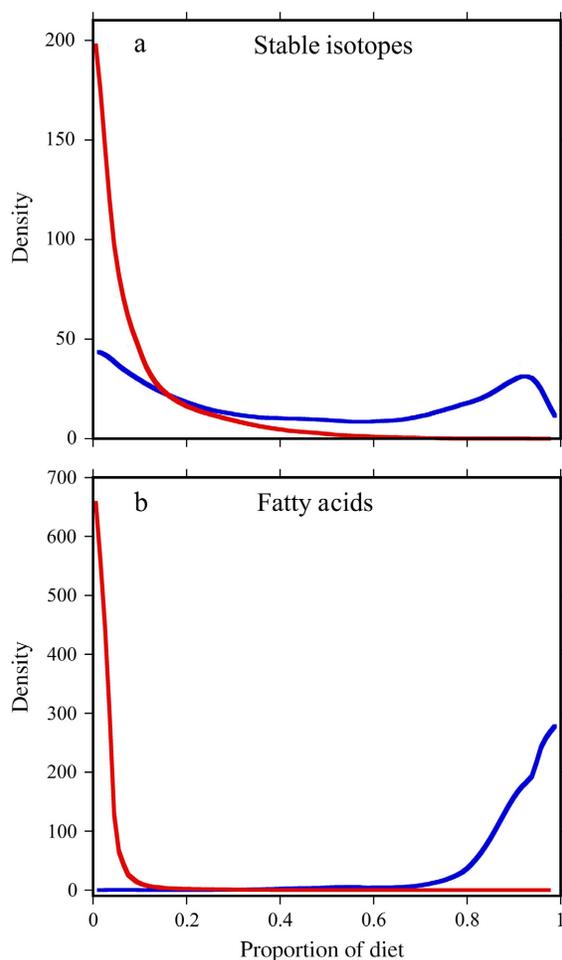


Fig. 5. The posterior distributions for the stable isotope (a) and fatty acid (b) analyses with known trophic modification. The results for SIAR and MixSIR are pooled in these plots. The blue curve depicts the outcomes for the macroalgae that comprised 100% of the isopod diet, and the red curve is the average output for the six macroalgae that were not consumed.

resource polygon, a situation common in real-world data. Because of this internal clustering, the seven resource polygons we considered had the same surface area as a polygon that only included three of these potential resources, that is, *Smithora*, *Mazzaella*, and *Fucus*. The complexity of the polygon we considered further constrained Bayesian mixing-model performance. The most obvious solution to this conundrum is to lump resources to simplify the structure of the polygon. However, in the present case, lumping resources with similar SI ratios would also entail

lumping together macroalgae with very different phylogenies, ecology, and even expected isopod diet preferences (Galloway et al. 2014a), which would greatly complicate the interpretation of any outcomes for an aggregated resource polygon.

Our mixing-model analyses of isopod resource utilization was based on all 26 FAs quantified. We also used NMDS to simplify the dimensionality of these data so that they could be depicted in bivariate space. The bivariate polygon based on the NMDS classification of the macroalgae and isopod FA profiles showed much better properties than the corresponding SI-based polygon. Firstly, in this classification, the animals fed algae from different phylogenetic groups separated very strongly as expected based on prior analyses of many of these same macroalgae (Galloway et al. 2012). Secondly, the resource polygon had a large surface area and five of the seven macroalgae helped define the outer boundaries of the polygon. Without correcting the samples for trophic modification, all of the isopod samples fell within the resource-mixing polygon. Additionally, all of the isopods fell near their

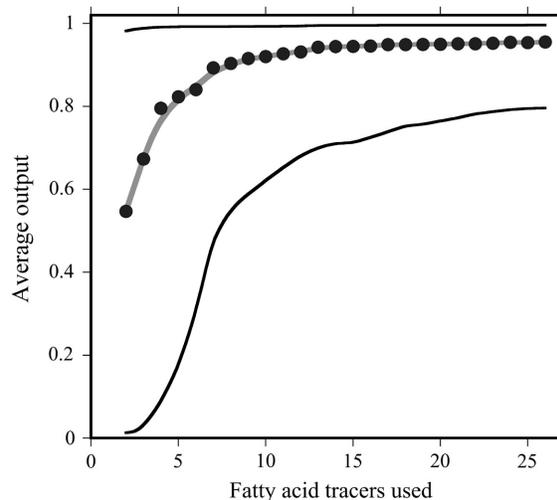


Fig. 6. The results of the tracer number experiment. The 26 tracers were ranked from the most to least variable between treatments, and then, a series of model runs were carried out that successively added additional fatty acid tracers starting with the most variable. The central thick gray trend line is the average model output. The thinner black outer lines are the 2.5% and 97.5% credibility intervals.

monospecific diets but also within the inner core of the resource polygon. Similar to past studies with *Daphnia* (Brett et al. 2006, Taipale et al. 2011), this outcome shows the FA profiles of the isopods were strongly influenced by their diets but that isopods in general had some common features to their lipid composition that were independent of diet. For example, when the macroalgal diets had low levels of the essential FA EPA, the isopods had a higher proportion of this FA. Conversely, when the isopods consumed red algae, which has a high proportion of EPA, they had less EPA in their tissues than their diets. Similar results were observed for the saturated FA palmitic acid (16:0) and the monounsaturated FA oleic acid (18:1ω9). The isopod FA profiles were very strongly influenced by their diets, but also tended to regress to the mean.

#### The “consumer-resource library”

Our results indicate that erroneous outcomes will likely result if diet-specific trophic modification is not directly accounted for even when considering greatly overdetermined mixing models. These results also showed that both SI and FA-based Bayesian mixing models perform poorly if a generalized “one-size-fits-all” trophic modification correction is used. To properly account for trophic modification, feeding experiments for each resource considered in the mixing polygon are required. Our results suggest it may be appropriate to use general FA trophic modification values within particular algal groups, for example, in this case for red and brown algae. These experiments are time intensive and are only possible for organisms that can be reared in laboratory conditions. In particular, it is important that the consumers considered appreciably increase their mass (e.g., by at least a factor of two to four) and that feeding trials are run long enough to enable tissue turnover, so that consumer biochemical composition at the end of the experiment reflects the test diets and not the initial conditions (Fry and Arnold 1982, Galloway et al. 2015).

The present results, and similar studies (Galloway et al. 2014a, b, 2015), indicate trophic biomarker mixing-model analyses should be based upon directly determined consumer biomarker trophic modification obtained in feeding trials. There are, however, legitimate challenges to this approach:

For example, many consumers, especially slow growing vertebrates, are difficult or impossible to keep in long-term controlled feeding trials. Despite this challenge, we believe the onus is on the analyst to take an approach that is meaningful, not just an approach that is based on conventional practices. Moving forward, we offer the following suggestions for advancing the field of trophic inference using biomarkers:

1. Despite the challenges, feeding trials are necessary. These experiments can require considerable time, but if researchers run experiments with different model taxa, over time we will develop diverse “consumer-resource” biomarker libraries for diverse species. For example, the necessary feeding trial data are already available so that analysts could use the FA-based mixing model to infer *Daphnia* diets in most lakes (Galloway et al. 2015).
2. Researchers should leverage the fact that zoos and aquaria often maintain organisms for long periods of time on consistent diets. Even if pure diets cannot be maintained, the measured trophic biomarker modification by captive animals could be compared with general trophic modification values.
3. When feeding trials are not feasible, it may be possible to make reasonable assumptions about biomarker trophic modification based on other experiments from related taxa. This may be particularly relevant for FA biomarkers for invertebrates and fish that are reared for aquaculture. These sources may provide a more meaningful proxy of a given taxon’s FA trophic modification than guesses or the assumption of no modification at all.
4. If it is not possible to run new feeding trials or glean data from the existing literature, analysts should test the sensitivity of their model results to a range of assumptions about unknown trophic modification factors.
5. Much more research is needed on the sensitivity of an organism’s “consumer-resource” library to variations in environmental conditions. Currently, these simplistic models have been based upon the biomarker profiles of experimental organisms kept in consistent temperatures and pure diets. These idealized conditions are quite different than what consumers typically experience in the field.

## CONCLUSIONS

The results of this study provide direct experimental evidence showing that Bayesian mixing models do not necessarily solve the underdetermined mixing-model constraint (e.g., Boecklen et al. 2011). Moreover, our experiments clearly show that to get meaningful results, it is critically important to measure and directly account for trophic modification for the major food resources. We have performed a relatively straightforward analysis of animals fed monoculture macroalgal diets during long feeding trials in which the animals grew substantially. Information of this quality is rarely collected prior to analyses of the biomarker signatures of wild consumers. Most importantly, even under these idealized circumstances, no mixing model or tracer tested was impervious to the need for proper accounting of trophic modification. Even in highly overdetermined scenarios using FA tracers, both mixing models performed poorly when trophic modification was assumed based on averages across all diets. This indicates the likely futility of running FA mixing models without accounting for diet-specific trophic modification.

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