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METHODS

Rod M. Connolly · Michaela A. Guest · Andrew J. Melville · Joanne M. Oakes

Sulfur stable isotopes separate producers in marine food-web analysis

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Abstract Ecological applications of stable isotope analysis rely on different producers having distinct isotopic ratios to trace energy and nutrient transfer to consumers. Carbon (C) and nitrogen (N) are the usual elements analysed. We tested the hypothesis that producers unable to be separated using C and N would be separated by sulphur (S), by reviewing estuarine and marine food web studies using all three elements (total of 836 pairwise comparisons between producers). S had a wider range of values across all producers than C and N (S: 34.4, C: 23.3, N: 18.7‰), and a higher mean difference among producers (S: 9.3, C: 6.5, N: 3.3‰). We varied from 1 to 10‰ the distance producers must be apart to be considered separate. For each of these gap distances, S-separated producers tied on C and N in 40% or more of cases. Comparing the three elements individually, S had fewer tied pairs of producers for any gap distance than C or N. However, S also has higher within-producer variability. Statistical tests on simulated data showed that this higher variability caused S to be less effective than C for analysing differences among mean producer values, yet mixing models showed that S had the smallest confidence intervals around mean estimates of source contributions to consumers. We also examined the spatial and temporal scales over which S isotope signatures of the saltmarsh plant *Spartina alterniflora* varied. Differences between samples taken within tens of metres were smallest, but between samples hundreds of metres apart were as different as samples thousands of kilometres apart. The time between samples being taken did not influence S signatures. Overall, the use of S is recommended because it has a high probability of distinguishing the contribution of different producers to aquatic food webs. When two elements are employed, the combination of S and C separates more producers than any other combination.

Keywords Estuary · *Spartina* · Trophic ecology · Variability

R. M. Connolly (✉) · M. A. Guest · A. J. Melville · J. M. Oakes
School of Environmental and Applied Sciences, Griffith University,
Gold Coast Mail Centre,
PMB 50, 9726, Queensland, Australia
e-mail: r.connolly@griffith.edu.au
Tel.: +61-7-55528614
Fax: +61-7-55528067

R. M. Connolly · M. A. Guest
The Cooperative Research Centre for Coastal Zone, Estuary and Waterway Management,
4068 Indooroopilly, Queensland, Australia

R. M. Connolly · M. A. Guest · J. M. Oakes
Centre for Aquatic Processes and Pollution, Griffith University,
Gold Coast Campus,
Queensland, Australia

Introduction

Analysis of naturally occurring stable isotope ratios of carbon (C), nitrogen (N) and sulfur (S) is commonly used to trace both the fate of organic matter and the ultimate source of energy (i.e. primary production) in aquatic systems. Typically, the first step in food web studies is to analyse the isotopic signature of a plant or other potential food source. This signature is the ratio of the rare, heavy isotope (e.g. ^{13}C, ^{15}N or ^{34}S) to the common, lighter isotope (e.g. ^{12}C, ^{14}N or ^{32}S), relative to international standards (Peterson and Fry 1987). The producer signature is then compared to those of consumers, which take on the isotopic signature of their food source. Stable isotope analysis differentiates between food that is assimilated and that which is merely ingested, giving it an advantage over other methods (e.g. gut contents analysis) in elucidating trophic dynamics.

Natural abundance stable isotope analysis using a single element cannot, however, determine the importance of different food sources to food webs where the isotopic signatures of potential sources are similar. To overcome this problem, two elements (typically C and N) are commonly employed to increase the chance of separating sources (Melville and Connolly 2003). Even where two

elements are used, the dietary contributions of some producers in aquatic systems remain unresolved where sources have the same isotopic signatures for both elements or where variation in signatures leads to an inability to distinguish between available producers (e.g. Loneragan et al. 1997).

The addition of S as a third element has been used in an attempt to determine the importance of potential food sources unable to be distinguished using a dual element approach (Peterson et al. 1985; Loneragan et al. 1997). Fry et al. (1982) identify four main sources of S available to estuarine plants. S present in the water column is generally in the form of sulfates and is isotopically enriched (~+20‰). Sedimentary sulfides formed by bacterial reduction of sulfates in anaerobic sediments are isotopically lighter (~−24‰). Porewater sulfates are more variable and can be more enriched than water column sulfates (~+60‰), or more depleted, if formed as a result of reoxidisation of sedimentary sulfides. For emergent or intertidal plants, rainwater sulfates with signatures from +2 to +16‰ are also a potential source of S (Fry et al. 1982). Producers utilising different sources of S therefore have different signatures. This allows discrimination between, for example, benthic and pelagic producers, such as saltmarsh plants and phytoplankton in estuarine studies (Peterson et al. 1986; Michener and Schell 1994).

In early aquatic trophic studies using stable isotopes, C alone or C and N were the most commonly used elements (e.g. Macko and Estep 1984; Zieman et al. 1984; Couch 1989; Harrigan et al. 1989), with S also being used in a small number of cases (e.g. Fry 1983; Peterson et al. 1985; Peterson and Howarth 1987; Fry 1988). Although S was proposed as likely to be able to differentiate sources unable to be separated using C and N (e.g. Harrigan et al. 1989; Peterson 1999), its use was limited by the laborious analytical techniques required to isolate the element from its matrix. S analysis was not automated and generally involved oxidising S to sulfate in solution. The sulfate was then precipitated as barium sulfate using a weak barium chloride solution, allowing the sample to be analysed in a mass spectrometer (Lajtha and Michener 1994). More recently, interest in S as an additional tracer to distinguish isotopic signatures amongst potential food sources has increased as the level of automation of S analysis has improved (Monaghan et al. 1999; Fry et al. 2002). A number of studies have reported that different producers have distinct S signatures (e.g. Peterson et al.1985; Moncreiff and Sullivan 2001), but the effectiveness of S in separating producers unable to be separated using C and N has not been demonstrated, and concern has been raised that variation in producer S signatures may limit their usefulness in food web studies (e.g. Stribling et al. 1998).

The purpose of this study was to:

1. Test whether S, as an additional tracer, discriminates between pairs of producers unable to be separated using C and N.
2. Contrast the ability of S to separate producers whose C and N isotopic signatures are tied with that of: (i) C, when N and S are tied, and (ii) N, when C and S are tied.
3. Determine which combination of two elements (out of C, N and S) best achieves isotopic separation between producers.
4. Assess the effect of variation in producer signatures on the ability of C, N and S to separate producers in food web studies.
5. Measure the spatial and temporal scales at which variation in S occurs in an estuarine producer.

Materials and methods

Separation of isotopic signatures of producers

We searched for all papers between 1978 and 2002 (inclusive) that reported stable isotope values of C, N and S for primary producers in marine and estuarine environments. Only those reporting values for all three elements for at least two producers were included. A total of 14 papers satisfied these specifications (Table 1). The stable isotope signatures of C, N and S of each producer that had a value for all three elements were extracted from each paper. Data were taken from tables where supplied, otherwise from figures.

Firstly, we tested the hypothesis that S would split two producers not able to be separated using C and N isotopic signatures. We tested this hypothesis for different gap distances, from 1 to 10‰. For example, using a gap of 1‰, two producers were considered tied (unable to be separated) when the difference between their C signatures was <1‰ and the difference between their N signatures was also <1‰. To split this tie, the difference between the S signatures of the two producers would have to be >1‰. Within each paper we determined the percentage of times that S would split producers tied on C and N, for each gap distance. This was done for every possible pair of producers within each paper. The comparisons were restricted to within a paper to better test the hypothesis that the use of S would help separate producers in a particular study.

We then tested which element produced fewer ties by repeating the above process looking for situations in which N separated producers tied on C and S, and C separated producers tied on S and N.

Table 1 Literature from which C, N, and S isotope data were extracted, showing the number of producer species for which data were obtained, and number of pairwise comparisons possible

Study	No. producer species	No. comparisons
Chanton and Lewis (1999)	4	6
Currin et al. (1995)	9	36
Deegan et al. (1990)	3	3
Deegan and Garritt (1997)	13	82
Kwak and Zedler (1997)	17	136
Loneragan et al. (1997)	4	6
Machás and Santos (1999)	8	28
Moncreiff and Sullivan (2001)	19	171
Newell et al. (1995)	17	136
Peterson and Howarth (1987)	7	21
Peterson et al. (1985)	3	3
Sullivan and Moncrieff (1990)	3	3
Wainright et al. (2000)	20	190
Weinstein et al. (2000)	6	15

Effect of variation in isotopic signatures

Numerical analysis of isotope data in trophic studies takes two main forms: (1) statistical comparisons among producers [i.e. analysis of variance (ANOVA) testing], and (2) estimating contributions of sources to the diet of a consumer using mixing models. To examine the effect of variability on the ability of C, N and S to separate producers, we recorded the variance and sample size associated with each mean isotopic signature in each paper. The average variance and sample size were then calculated for C, N and S isotopic signatures of producers (and, for mixing model analysis, consumers, where these were included in papers). These values were used in the following analyses.

For each element, ANOVA tests were run on simulated data, using four producers, with mean values for the producers evenly separated. Three scenarios with different distances between means were tested (Table 3): small (1‰ for C and N, 2‰ for S, since the range for S is greater), medium (the mean gap distance from all pairwise comparisons, above), and large (the largest possible distance between means still allowing all values to lie within the reported range of signatures for that element). The ability of C, N and S to separate producers was evaluated using probability values and *F* statistics from the ANOVA tests.

For mixing model analysis, we used a recently developed model that incorporates producer and consumer variation and confidence intervals around mean source contribution estimates (Phillips and Gregg 2001). For each element, two-source mixing models were run on simulated data. In this case, two scenarios with different distances between producer means were run (Table 4), equivalent to the medium distance described for ANOVA, above (using mean gap distance), and a large distance (double that used in the ANOVA above, since only two means are involved here). For the mixture (consumer), a mean value midway between the two producers was used in each case. The mean contribution estimated for both sources was thus 50%. The usefulness of the three elements was evaluated by comparing confidence intervals around the estimates of source contributions, with smaller intervals considered better.

Variation in space and time

To investigate the temporal and spatial scales at which variability in S isotopic signatures occurs, all papers presenting S values for producers from 1978–2002 were considered (regardless of whether C and N values were obtained). The mean S value of each producer for every sample was recorded, at the lowest level of resolution presented in papers (i.e. before any grouping of different sites or times had been done by authors). The intention was to compare variability within a producer species, to avoid confounding with differences among species. Enough data for useful analysis existed only for the saltmarsh plant *Spartina alterniflora*, and for this species, values were combined from all 13 papers in which they occurred. We then determined how far apart in space and time each sample was from each other sample (Table 2). The following spatial categories, measured as linear distance (km) regardless of coastline shape, were used: 0.01 (i.e sites 10s of metres apart), 0.1 (100s of metres apart), 1.0, 10, 100 and 1 000 (i.e. greater than 1,000 km apart). The temporal scale was divided into different intervals between sampling times (in months): <1 month (i.e. samples taken within 30 days of one another), <3 months (between 30 and 90 days apart), <6, and <12 months. We also categorised samples, regardless of the year of sampling, into seasons and into calendar months (n.b. all *S. alterniflora* values were from the northern hemisphere), and into calendar year, regardless of month. Within each spatial and temporal category, the gap distance between all possible pairs of S values was determined.

We also searched for opportunities to separate spatial and temporal differences, where *S. alterniflora* samples were collected from different places at the same time, or different times in the same place. The only S isotope data that offered such an opportunity were from Stribling et al. (1998). We used the spatial and temporal categories described above to evaluate these data.

Table 2 Literature sources of data used to analyse spatial and temporal scales of variability in *Spartina alterniflora*, either within a study or in combination with other studies

Study	Spatial scales (km)						Temporal scales						
							Within intervals (months)						
	0.01	0.1	1	10	100	1,000	1	3	6	12	Season[a]	Calendar month[a]	Calendar year[b]
Cornwell et al. (1990)				×	×	×							
Currin et al. (1995)		×			×	×	×	×	×	×	×	×	×
Deegan et al. (1990)					×	×							
Deegan and Garritt (1997)		×	×	×	×	×		×			×		×
Fry et al. (1982)	×				×	×							
Kwak and Zedler (1997)						×							
Peterson et al. (1985)				×	×	×							
Peterson et al. (1986)	×			×	×	×							
Peterson and Howarth (1987)	×				×	×					×	×	
Stribling et al. (1998)	×	×	×	×	×	×	×	×	×	×	×	×	×
Sullivan and Moncreiff (1990)						×							
Wainright et al. (2000)	×		×	×	×	×	×	×			×	×	×
Weinstein et al. (2000)				×	×	×						×	

[a] regardless of year

[b] regardless of month

Results

Separation of isotopic signatures of producers

A total of 836 pairwise comparisons were examined from the 14 papers (mean 60 comparisons per paper). S was clearly superior at separating sources, with the percentage of tied cases (8–66%) considerably lower than for C (29–79%), which had lower values than N (24–92%) for all gap distances except 1‰ (Fig. 1A). S had a wider range of values (−14.0 to +20.4‰, range 34.4) over all producer values than C (−31.8 to −8.5‰, range 23.3) or N (−1.4 to +17.3‰, range 18.7). The mean difference between producers across all pairwise comparisons was also greater for S (9.3‰±0.2) (mean±SE) than for C (6.5‰±0.2) or N (3.3‰±0.1).

At a gap distance of 1‰, C and N separated more tied producer pairs (75% and 90%, respectively) than S (67%). However, for all other gap distances, S separated pairs of producers tied on C and N in about 40% or more of cases (Fig. 1B). This was clearly superior to C, which separated pairs tied on N and S in only about 30% of cases for gaps greater than 2‰. N was even poorer at breaking ties, separating pairs tied on C and S in only 15% or fewer cases for gaps >2‰.

The combination of elements separating the highest proportion of producer pairs at all gap distances was that of C and S. For C and S, 56–98% of cases were separated for the range of gap distances (Fig. 1C), whereas the combination of N and S separated a lower proportion of cases (40–97%). C and N separated the fewest cases for all gap distances (14–92%).

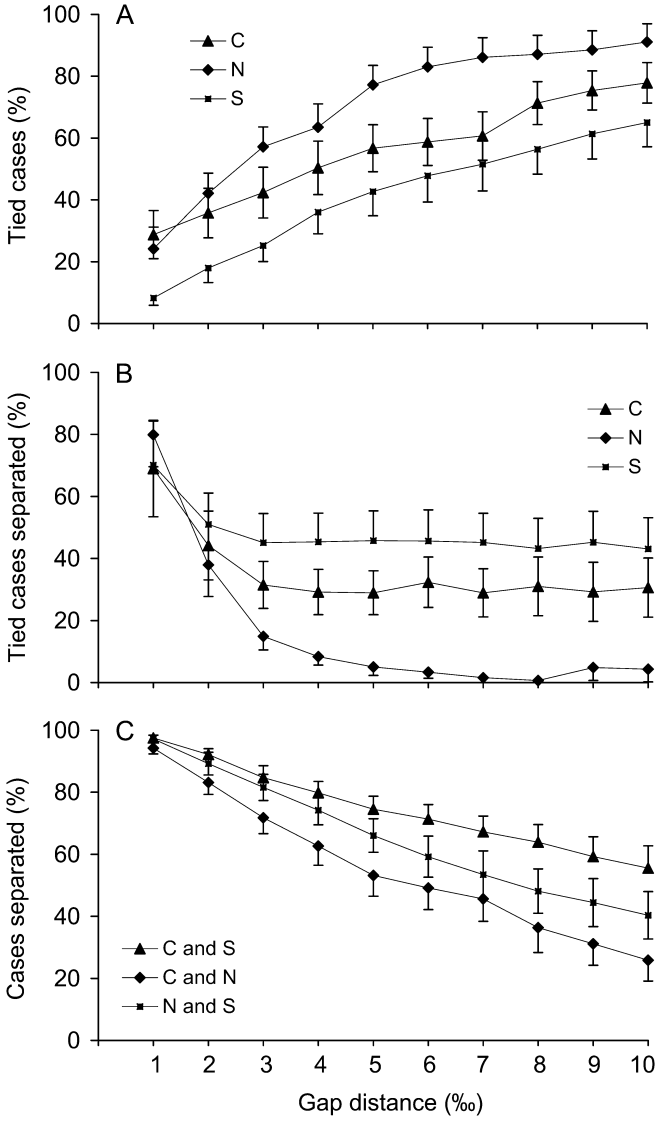

Fig. 1 Ability of C, N and S to separate producers, showing percentage of cases (producer pairs): **A** tied using a single element, **B** tied for two elements but separated by a third (lines labelled by third element), and **C** separated using different combinations of two elements, all for gap distances from 1 to 10‰, combining data from all papers (mean±SE)

Effect of variation in isotopic signatures

The average variance in producer signatures was greatest for S (8.5‰), intermediate for N (2.4‰), and lowest for C (1.0‰). ANOVA tests of simulated mean values for four producers found significant differences among means for all gap distances (small, medium and large) for C (Table 3). Tests on S were significant for medium and large gap distances, but not quite for the smallest gap, and the pattern was the same for N. *F* statistics were ordered, from largest to smallest, as C, S and then N (Table 3), demonstrating a trend in ability to separate producer means in that order.

Using two-source mixing models, S gave the smallest confidence intervals around the mean estimated contribution for both the medium and large gap distances (Table 4). C had the next best confidence interval for medium gap distances, but N was next best for large gaps.

Table 3 ANOVA results (*F*-statistics and *P*-values) on simulated data with four producer means evenly spaced at different gap distances. Producer sample size (*n*) and variance (s^2) are means obtained from the literature. The three gap distances are small, medium and large, as described in the text

Element	*n*	s^2	Gap (‰)	*F*	*P*
C	6	1.0	1.0	10	<0.001
			6.5	422	<0.001
			7.0	490	<0.001
N	3	2.4	1.0	2	0.171
			3.3	22	<0.001
			6.0	75	<0.001
S	4	8.5	2.0	3	0.064
			9.3	67	<0.001
			11.0	94	<0.001

Table 4 Results of two-source mixing models (shown as confidence intervals, CI, around an estimated mean contribution for a producer of 50%). Producer and consumer sample sizes (n) and variances (s^2) are means obtained from the literature. The two gap distances are medium and large, as described in the text

Element	Consumer		Producer			CI
	n	s^2	n	s^2	Gap (‰)	
C	3	1.2	6	1.0	6.5	0.91
					14.0	0.42
N	3	0.8	3	2.4	3.3	1.00
					12.0	0.33
S	2	1.2	4	8.5	9.3	0.77
					22.0	0.32

Variation in space and time

The difference in S isotope values for *S. alterniflora* between pairs of sample means was smallest at the smallest scale (sites 10s of metres apart) and intermediate at the 1-km scale (sites 1,000s of metres apart); differences at all other scales, including the 0.1-km scale, were larger and were similar to each other (Fig. 2A). At the three largest scales, median differences were not particularly high but the upper end of the range was higher than at smaller scales, indicating an occasional pair of samples with very different values. The differences between pairs of samples taken at different time intervals were very similar, as were samples taken in the same season, calendar month or calendar year (Fig. 2B).

When S isotope values from Stribling et al. (1998) were used to separate spatial and temporal effects, we were again unable to determine any differences due to the time interval between sampling (Fig. 3). The spatial pattern was the same as that described above, with larger differences between means at the 0.1-km scale than at the 1-km scale, regardless of the time interval between sampling (Fig. 3).

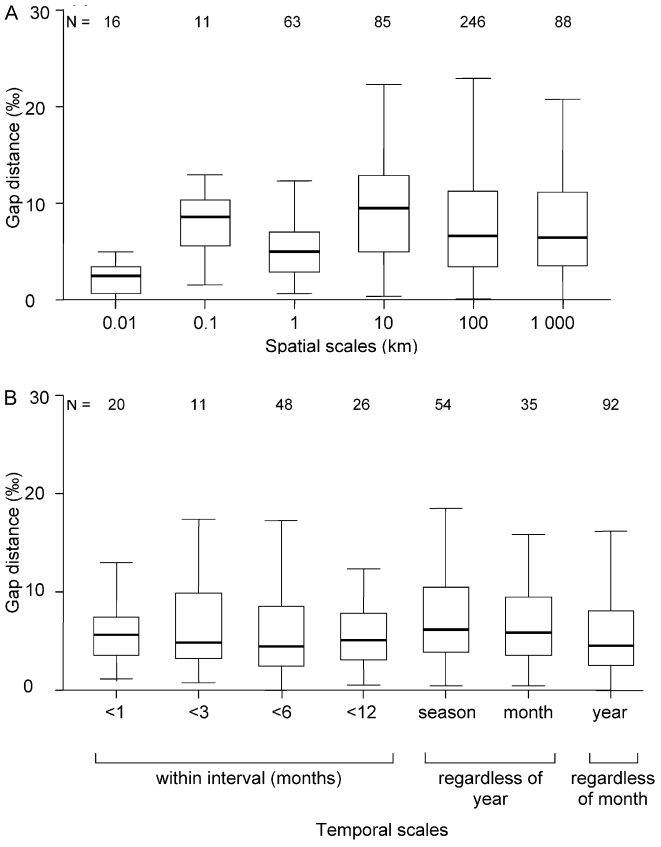

Fig. 2 Variability in sulfur signatures of live *Spartina alterniflora* samples at **A** different spatial scales, and **B** different temporal scales. *Box plots* show median, 25th and 75th percentiles, and range

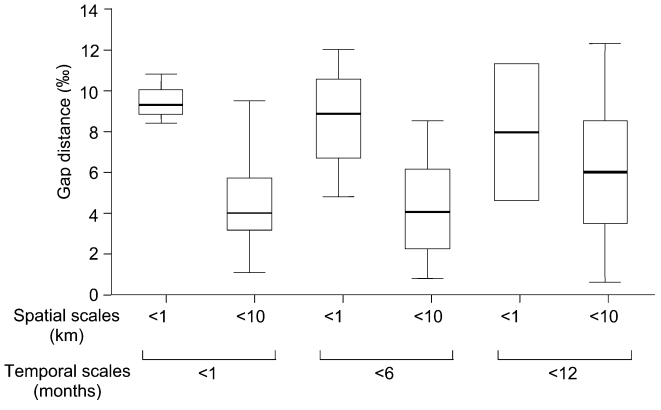

Fig. 3 Variability in sulfur signatures of live *S. alterniflora* showing spatial and temporal components separated (data from Stribling et al. 1998). *Box plots* show median, 25th and 75th percentiles, and range

Discussion

Separation of isotopic signatures of producers

These results support the hypothesis that where the isotopic signatures of two producers are not able to be separated using C and N, S as an additional tracer frequently discriminates between those signatures. Furthermore, the discriminatory capacity of S appears to be due to characteristics peculiar to S isotopes rather than simply the addition of a third element. Of the 836 comparisons amongst producer isotopic signatures, S tied less than C or N. Typically this is because for any pair of producers, there is a greater difference between the mean isotopic signatures for S than for C or N. This is most likely due to the large differences in the ratios of the heavy and light S isotopes among sources used by producers. Isotopic signatures of producers remain within ~4‰ of their source of S (Mekhtiyeva et al. 1976; Chukhrov et al. 1980; Peterson and Fry 1987), and isotopic differences amongst producers are considered to be more likely a function of sources than of diffusive or metabolic effects (Fry et al. 1982; Trust and Fry 1992). For example, producers that predominantly utilise seawater sulfates tend to be enriched (e.g. microalgae and phytoplankton ~ +18‰, Thode 1991) while those utilising sedimentary sulfides are more depleted (e.g. marsh plants −10 to +5‰, Thode 1991; Kharlamenko et al. 2001). Moncreiff and

Sullivan (2001) have also proposed differences in source S as the reason for the distinction between the S isotopic signature of the seagrass *Halodule wrightii* (+11‰) and its associated algal epiphytes (+18‰).

Differences between isotope values of C are on average less than those of S, but are consistently greater than those of N. Likely explanations for different C isotopic ratios among producers are differential utilisation of $HCO_3$and CO_2 (Coffin et al. 1989; Farquhar et al. 1989) and the differing fractionation of C through either C_3, C_4 or CAM photosynthetic pathways (O'Leary 1988; Peterson 1999). In most studies of aquatic food webs, N is likely to be of least use in separating the contribution of different producers. The usefulness of N lies in its relatively large degree of fractionation across trophic levels, which can help to elucidate trophic levels of consumers (Fry et al. 1999; Pinnegar and Polunin 2000).

The higher likelihood of S separating any two producers results in S being better able to discriminate between producers tied on C and N than C can for producers tied on S and N or N can for those tied on C and S. Moreover, S proves a useful discriminator between tied isotopic signatures across all gap distances.

Effect of variation in isotopic signatures

Variability of isotope signatures within a sample of a single producer species is greater on average for S than for C and N. Does this higher variation in S outweigh the advantage of a typically greater gap distance among producers? In ANOVA tests among simulated means with typical gap distances, variances and sample sizes for each element, all elements proved to be useful in separating producers. However, C was best across each gap distance used, followed by S and then N. Mixing models designed to measure the potential contribution of sources to the diet of a consumer showed that S had the smallest confidence intervals around mean contribution estimates, for both gap distances used. Either C or N were next best depending on the gap distance involved. Interestingly, for these scenarios based on realistic values for means, variances and sample sizes for producers and consumers, even S had surprisingly wide confidence intervals. For example, in the scenario using mean gap distance, the estimate of a producer contribution based on S lay somewhere between 11.5 and 88.5‰ (mean estimate 50‰, CI 77‰, from Table 4). Estimates based on C or N had even wider confidence intervals. In summary, S gives the tightest confidence limits in mixing model results but C is superior for separating producer means statistically.

Variation in space and time

Differences in S signatures of *S. alterniflora* were found to be as great between samples 100s of metres apart as between samples 1,000 s of km apart. This pattern occurs because *S. alterniflora* samples from different parts of a single estuary have signatures far apart. The same differences occur within other estuaries, yet the overall S signatures of each estuary are approximately the same. The differences in *S. alterniflora* signatures within an estuary probably result from differences in organic load and therefore S characteristics of sediment (Stribling et al. 1998). We could not detect any differences in S signatures of *S. alterniflora* sampled at different times.

Conclusions and recommendations

S isotope signatures for producers tend to be further apart than those for C or N, and although variation within producer samples is also higher, S remains a very useful element for marine food-web analysis. We suggest increased focus on measuring and understanding variability in S isotope signatures in marine food webs. There is genuine scope for taking better account of variability and thus reducing confidence intervals in estimates of source contributions to consumers.

The use of S in determining the ultimate primary food sources of consumers is also advantaged by its low levels of fractionation across trophic levels (Peterson et al. 1985; Peterson and Fry 1987), although these levels have recently been shown to vary depending on food quality (McCutchan et al. 2003). Whilst fractionation such as that in N can be useful for elucidating trophic levels, it often confounds attempts to use that element for determining food sources. Elements such as S that track assimilation more conservatively are better for separating sources.

The automation of S isotope analysis of ecological samples is increasing both the breadth of food web studies in which S can be employed and the levels of replication that can be used. However, on a cautionary note, sampling and analysis artefacts are less well understood for S than for C or N. Improved preparation and analytical techniques (e.g. Hsieh and Shieh 1997, Fry et al. 2002) are being developed but need to be more widely tested and used to give rigour to the use of S in food web studies.

We conclude that in many respects S should be the element of choice in marine food web studies, and that S and C should be employed where two elements can be used. However, if C and N have been used, our results indicate that S is likely to split any remaining ties.

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