



Why aquatic scientists should use sulfur stable isotope ratios ($\delta^{34}\text{S}$) more often

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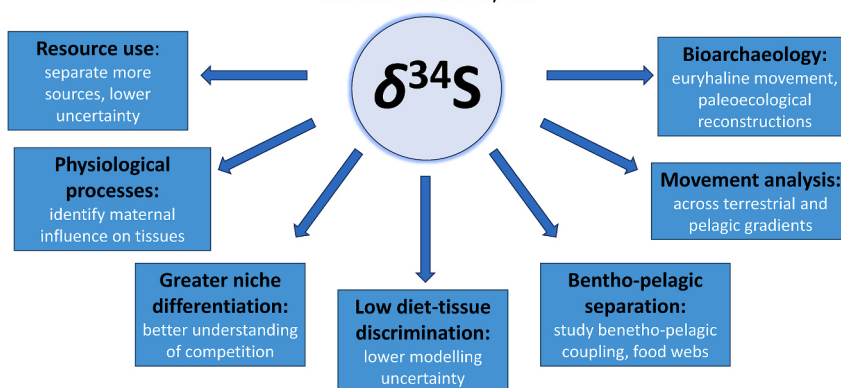
HIGHLIGHTS

- The use of sulfur stable isotopes has lagged behind other stable isotopes.
- We explore the reasons why the use of this stable isotope is less frequent.
- We analyse real-world stable isotope datasets with and without sulfur to demonstrate the effects of excluding it.
- Incorporating sulfur isotopes changes how results are interpreted and its broader use would benefit aquatic sciences.

GRAPHICAL ABSTRACT

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ABSTRACT

Over the last few decades, measurements of light stable isotope ratios have been increasingly used to answer questions across physiology, biology, ecology, and archaeology. The vast majority analyse carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopes as the 'default' isotopes, omitting sulfur ($\delta^{34}\text{S}$) due to time, cost, or perceived lack

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SIA
Mixing models
Ecology
Niche space
Isoscapes
Bioarchaeology

of benefits and instrumentation capabilities. Using just carbon and nitrogen isotopic ratios can produce results that are inconclusive, uncertain, or in the worst cases, even misleading, especially for scientists that are new to the use and interpretation of stable isotope data. Using sulfur isotope values more regularly has the potential to mitigate these issues, especially given recent advancements that have lowered measurement barriers. Here we provide a review documenting case studies with real-world data, re-analysing different biological topics (i.e. niche, physiology, diet, movement and bioarchaeology) with and without sulfur isotopes to highlight the various strengths of this stable isotope for various applications. We also include a preliminary meta-analysis of the trophic discrimination factor (TDF) for sulfur isotopes, which suggest small (mean -0.4 ± 1.7 ‰ SD) but taxa-dependent mean trophic discrimination. Each case study demonstrates how the exclusion of sulfur comes at the detriment of the results, often leading to very different outputs, or missing valuable discoveries entirely. Given that studies relying on carbon and nitrogen stable isotopes currently underpin most of our understanding of various ecological processes, this has concerning implications. Collectively, these examples strongly suggest that researchers planning to use carbon and nitrogen stable isotopes for their research should incorporate sulfur where possible, and that the new 'default' isotope systems for aquatic science should now be carbon, nitrogen, and sulfur.

1. Introduction to sulfur isotopes

An understanding of the ecology and physiology of organisms is at the core of conservation and management (Horan et al., 2011). Knowing the drivers of animal movement and their role within food webs can help limit negative human interactions as well as predict how a changing planet will impact ecosystems. While many tools such as direct observation, tagging and diet analyses are available to scientists to understand animal ecology, only stable isotopes can address numerous ecological aspects concurrently and at relatively low cost. Consequently, stable isotope ecology as a field has expanded rapidly since the 1980s (Peterson and Fry, 1987), being widely adopted by ecologists (West et al., 2006). The vast majority of studies using stable isotopes in ecology rely on carbon and nitrogen isotopes exclusively (Bird et al., 2021; Silverman et al., 2022), despite numerous other ecologically-relevant isotopes being available to researchers. This may potentially limit the utility of many stable isotope studies and increases the likelihood that resulting analytical interpretations are incorrect.

While carbon and nitrogen isotopes are prevalent in natural tissues, there are many other common naturally-occurring stable isotopes that are of use to understand life. For example, oxygen isotopes are used to assess temperature differences (Shemesh et al., 1992), and can be used to trace movement or historical climate patterns (Shackleton, 1967). Hydrogen isotopes in animal tissues relate to water absorption and can be used to interpret physiology and movement (Hobson, 2019; Vander Zanden et al., 2016). Assessing these other isotopes typically requires specific use-cases since they come at much greater cost to analyse relative to carbon and nitrogen, and sample processing or interpretation requirements can make them less attractive than bulk isotope analysis of carbon and nitrogen. As a result, the analysis of isotopes other than carbon and nitrogen makes up just a fraction of stable isotope studies.

Sulfur stable isotopes ($\delta^{34}\text{S}$) have been used since the 1970s for ecological applications with interest accelerating in the 1980s (Fry, 1988; Fry et al., 1982; Matrosov et al., 1975; Mekhtiyeva et al., 1976; Peterson and Fry, 1987). Their incorporation, in theory, can add substantial value to interpretations. The primary mechanism that modifies sulfur isotope ratios is bacterial fractionation of inorganic sulfur compounds, usually occurring in anoxic sediments (Fry et al., 1986; Rees, 1973). In practical terms, this means that sediment-bound food webs have lower $\delta^{34}\text{S}$ values, while pelagic systems not impacted by these anoxic processes have higher values (Szpak and Buckley, 2020). Thus, where an ecosystem of interest is connected to sources of low and high $\delta^{34}\text{S}$ values, the incorporation of this isotope may be especially beneficial. This includes pelagic and benthic ecosystems (Szpak and Buckley, 2020), coastal and nearshore habitats (Connolly et al., 2004), freshwater ecosystems linking terrestrial and estuarine environments (Guiry et al., 2021), and even in deep hydrothermal vent communities exposed to chemoautotrophic primary production (Fry et al., 1983; Reid et al., 2013). Thus, sulfur isotopes are likely the most useful additions to

studies when sources in the ecosystem of interest differ greatly in $\delta^{34}\text{S}$ values, allowing mixing calculations with higher fidelity than other systems that may have less natural variation between sources.

Many aspects of organic sulfur compounds make them attractive for use in aquatic studies, including the apparent lack of or very low trophic fractionation in comparison to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Like nitrogen isotopes, sulfur isotopes are transferred through dietary proteins in the form of the "essential" amino acid methionine (Florin et al., 2011; Phillips et al., 2021). Despite its potential in many ecological and biological applications, the use of $\delta^{34}\text{S}$ has not proliferated like $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Fig. 1), possibly because of perceived legacy issues with analytical chemistry separations (e.g., accidental oxidation/fractionation of S atoms) and measurement (e.g., poor separation on GC columns), which were associated with requiring larger samples, greater analytical cost, and/or times.

Recent advancements (~10 years) in isotope instrumentation have significantly lowered analytical barriers to measuring $\delta^{34}\text{S}$ values simultaneously with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Most commonly, scientists have relied on an Elemental Analyzer (EA) to combust analytes to SO_2^+ before online measurement in an Isotope Ratio Mass Spectrometer (IRMS). To compensate for the gas' polarity and resulting broad peaks on gas

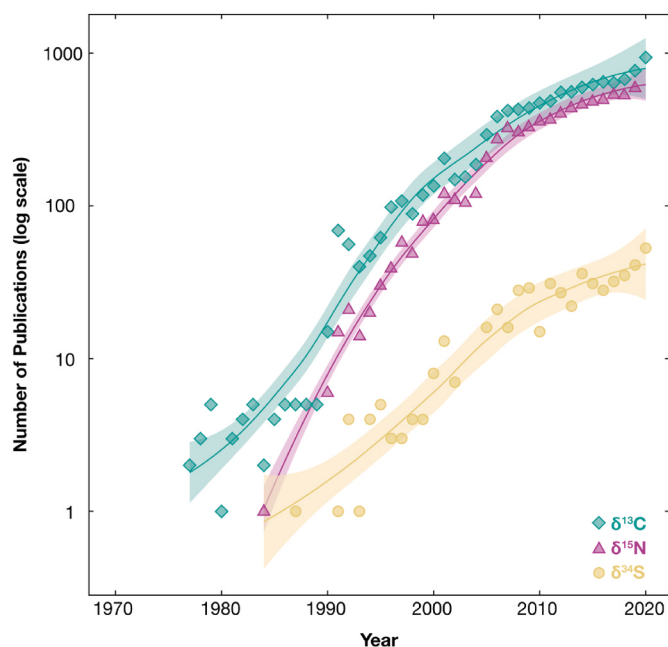


Fig. 1. Number of publications per year with $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ or $\delta^{34}\text{S}$ values in the key terms, via Web of Science excluding geology and geochemistry fields. Each isotope is overlaid with LOESS smoothing curves and 95% CI.

chromatography (GC) columns during separation, traditional measurements have required large sample sizes (~100s of ug of S). However, elemental analyzer modifications including “purge and trap” (Fourel et al., 2014), ramped GC columns (Phillips et al., 2021; Sayle et al., 2019), and dual GC column systems (Fry, 2007) have lowered sample sizes to as low as 1 μ g S (<1 mg of bulk tissue), while maintaining precision <0.2 ‰. Notably, more work is needed to fully optimize EA-IRMS systems to run simultaneous C, N, and S measurements, as in practice many labs analyse samples in C and S or C and N modes. Other emerging innovations in stable isotope geochemistry may solve this problem, although they are not yet ready for routine use by ecologists. Namely, MC-ICP-MS (Multi Collector Inductively Coupled Plasma Mass Spectrometry) and Orbitrap-MS offer the potential for high mass resolution for multiple isotope systems simultaneously and have initial promising demonstrations with sulfur-containing organic compounds (Amrani et al., 2009; Neubauer et al., 2018; Rodiouchkina et al., 2023). Nonetheless, these advancements have made the analysis of $\delta^{34}\text{S}$ values at lower cost, higher precision, and with mostly similar sample pre-treatments as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values.

Other ecological methodologies, such as those involving DNA techniques, have evolved rapidly in conjunction with improvements in analytical approaches and computing ability (Washburn et al., 2019). In contrast, the methods behind stable isotope analyses for life sciences have remained relatively constant outside of improvements in computational tools. Although C, H, O, N, and S isotopes are the canonical toolkit for aquatic ecologists, there has been an overreliance on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values alone because, rightly or wrongly, many feel that these two stable isotope ratios will suffice to answer research objectives. This is perhaps because there are few explicit study examples empirically testing how integrating $\delta^{34}\text{S}$ values relative to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values alone may benefit their particular case. Making use of real-world examples of stable isotope studies re-analyzed with and without $\delta^{34}\text{S}$ values, we provide justification to argue that the use of sulfur isotopic ratios should be as widespread as that of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. These will highlight the marked benefits that broader use of sulfur isotopic ratios could provide to scientists interested in using stable isotopes to answer a variety of ecological questions.

2. Ecology and niche space

Consumer resource use, movements, and trophodynamics can be investigated by considering the spread of a consumer's isotope values across multivariate space: its isotopic niche, the multivariate space where axes are isotopic values for elements and the isotopic composition of the tissues of an animal (Newsome et al., 2007). The size of the niche indicates the breadth of resources used, while niche overlap among co-occurring groups suggests a degree of potential competition (e.g. if resources are limited). To date, most studies have used $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values to generate 2-D isotope niche ellipses (Jackson et al., 2011), but the addition of a third tracer and axis to generate 3-D ellipsoids may help reveal individual specialisation in a population (Skinner et al., 2019) or a wider pool of resource use (Cybulski et al., 2022). The use of sulfur for isotopic niche analysis is not common, despite the availability of tools to examine 3-isotope niches in a similar fashion as 2 isotope niches (Swanson et al., 2015). Conceptually, ellipsoid volumes are still relatively new to isotopic niche analysis, but may better reflect the complexities of ecosystem function (Rossman et al., 2016), although their interpretation may be more nuanced than the traditional 2-dimensional format. This may discourage the broader use of 3-isotope (including sulfur) niche analysis, despite the many benefits that are identified.

In aquatic studies, including $\delta^{34}\text{S}$ values as a third tracer may be particularly useful due to the large range in $\delta^{34}\text{S}$ values across different water sources and aquatic primary producers (Connolly et al., 2004; Peterson and Fry, 1987). $\delta^{34}\text{S}$ values can therefore further delineate isotopic niches when $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values vary minimally. Aquatic projects that have used $\delta^{34}\text{S}$ values found, in many cases, that it revealed

new insights that were not present with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values exclusively. In coastal environments, sea turtle niches were more differentiated by $\delta^{34}\text{S}$ values than $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Weber et al., 2023), and $\delta^{34}\text{S}$ values drove the largest niche size separation in whales (Borrell et al., 2021). In estuarine systems, including $\delta^{34}\text{S}$ values rather than only $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ revealed more precise information on the habitat use of large marine mammals (Cani et al., 2023), a larger range of individual variation (Wilson et al., 2017), greater differentiation of large predatory shark niches (Seubert et al., 2019), and niche separation of adults and juvenile crabs (Bopp et al., 2023). In freshwater systems, apparent narrow isotopic niches of some species of fish with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values did not necessarily align with the variability of $\delta^{34}\text{S}$ values, allowing the detection of the effects of wet and dry seasons on the isotopic niche (Pool et al., 2017). Near hydrothermal vents, $\delta^{34}\text{S}$ values allowed the separation of different chemoautotrophic pathways (Suh et al., 2022), and large vs small mussel aggregations (Demopoulos et al., 2019). Thus, across many aquatic ecosystems, including $\delta^{34}\text{S}$ values can reveal novel insights for isotopic niche analysis.

To empirically demonstrate how incorporation of $\delta^{34}\text{S}$ values can benefit and change the interpretation of isotopic niche analysis, we re-analyse data from white muscle tissue from four groupers from North Malé Atoll, Republic of Maldives (*Aethaloperca rogaa*, *Anyperodon leucogrammicus*, *Cephalopholis argus*, and *C. miniata* from Skinner et al. (2019)). Isotopic niches were first generated for each species using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, and then, niches were re-generated with the addition of $\delta^{34}\text{S}$ values. When adding $\delta^{34}\text{S}$ values as a third tracer, the isotopic niches of *A. leucogrammicus* and *C. miniata* remained a similar size, while that of *A. rogaa* decreased and that of *C. argus* increased (Table 1). Despite the additional information a third tracer can convey, it may not always increase the relative size of a group's isotopic niche.

However, although isotopic niche size did not vary much, the extent of niche overlap was substantially reduced: mean overlap across all pairs was 64% when using only $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values but fell to 53% when $\delta^{34}\text{S}$ values were introduced (Table 2). Individually, isotopic niche overlaps among groupers were reduced by ~10–20% (Table 2). Only one species did not change (*C. miniata*), indicating that $\delta^{34}\text{S}$ values helped to separate their niche from the others and that they may be using a wider pool of resources than previously suspected. Furthermore, overlaps are often considered biologically significant when they are >60% (Matley et al., 2016; Zaret and Rand, 1971). Here, with ellipses there were 8 instances of significant overlap, but when $\delta^{34}\text{S}$ values were introduced, there were only 3. Clearly, $\delta^{34}\text{S}$ values can be fundamental for understanding the extent of resource competition among sympatric species in various settings, and the use of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values exclusively can overinflate the likelihood of competition occurring, as has been identified in other studies (Seubert et al., 2019). Where researchers are less familiar with the correct interpretation of isotopic niche overlap and how that may differ from ecological niche overlap (e.g., overlap does not necessarily equate to competition, Hette-Tronquart (2019)), the use of $\delta^{34}\text{S}$ values reduces the likelihood of this misinterpretation.

Table 1

Median isotopic niche based on 95% Bayesian Standard Ellipse Area ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and 95% Bayesian Standard Ellipsoid Volume ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, and $\delta^{34}\text{S}$) for four grouper species (*Aethaloperca rogaa* n = 22; *Anyperodon leucogrammicus* n = 20; *Cephalopholis argus* n = 22; and *C. miniata* n = 21) across North Malé Atoll, Republic of Maldives. NB: Values are scaled and mean-centred to allow for a direct comparison between metrics.

Species	Without $\delta^{34}\text{S}$	With $\delta^{34}\text{S}$
<i>A. rogaa</i>	0.99	0.80
<i>A. leucogrammicus</i>	0.11	0.10
<i>C. argus</i>	0.28	0.54
<i>C. miniata</i>	−1.39	−1.44

Table 2
Difference in the percentage of isotopic niche overlap between values calculated using ellipses ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and ellipsoids ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$).

	A. rogae	A. leucogrammicus	C. argus	C. miniata
A. rogae	–	–8 %	–10 %	6 %
A. leucogrammicus	–18 %	–	–12 %	2 %
C. argus	–18 %	–15 %	–	0.0 %
C. miniata	–22 %	–13 %	–17 %	–

3. Physiology

Stable isotopes are an effective non-lethal tool for studying animal physiology (Ehleringer et al., 1986; Gannes et al., 1998). Many of the processes governing diet-tissue isotopic discrimination are physiological, and therefore the degree of fractionation of a given isotope will reflect physiological processes. Stable isotopes can thus be used to study various animal physiological processes, including stress (Karlson et al., 2018), metabolism (Trueman et al., 2013, 2023), condition (Cherel et al., 2005; Feeney et al., 2024), and even gut microbial physiology (de Graaf and Venema, 2007).

The use of sulfur isotopes in this space is less commonplace, perhaps because our understanding of sulfuric incorporation pathways is less than for stable isotopes of C and N, which comprise the bulk of tissue mass. Unlike $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values that often reflect growth, in bivalves $\delta^{34}\text{S}$ values may be more correlated with sulfide detoxification activities than normal tissue turnover (Dattagupta et al., 2004), and in vestimentiferans (polychaete tube worms) found near black smokers, $\delta^{34}\text{S}$

values reflect those of highly variable environmental sulfides absorbed by symbionts (Becker et al., 2011). These factors, however, highlight that physiological processes impacting $\delta^{34}\text{S}$ values may be separate to those affecting $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values exclusively, which offers the potential to use this information to better understand physiological processes.

For species with high maternal investment such as intrauterine gestation, isotopic signatures of young-of-the-year individuals may not accurately reflect their own diet as tissue composition can partly, or largely originate from their mother's food sources (Olin et al., 2011; Raoult et al., 2022a). This is also likely to be not only species-specific (based on physiology) but also tissue-specific, with turnover rates influencing the amount of time that maternal signatures will be still found in juvenile tissues. Identifying when maternal stable isotope signatures are lost can be difficult in systems where multiple food resources are available to predators, and $\delta^{15}\text{N}$ values of prey can be high.

In this example that demonstrates how using $\delta^{34}\text{S}$ values can benefit physiological studies, Niella et al. (2021) used muscle tissue from

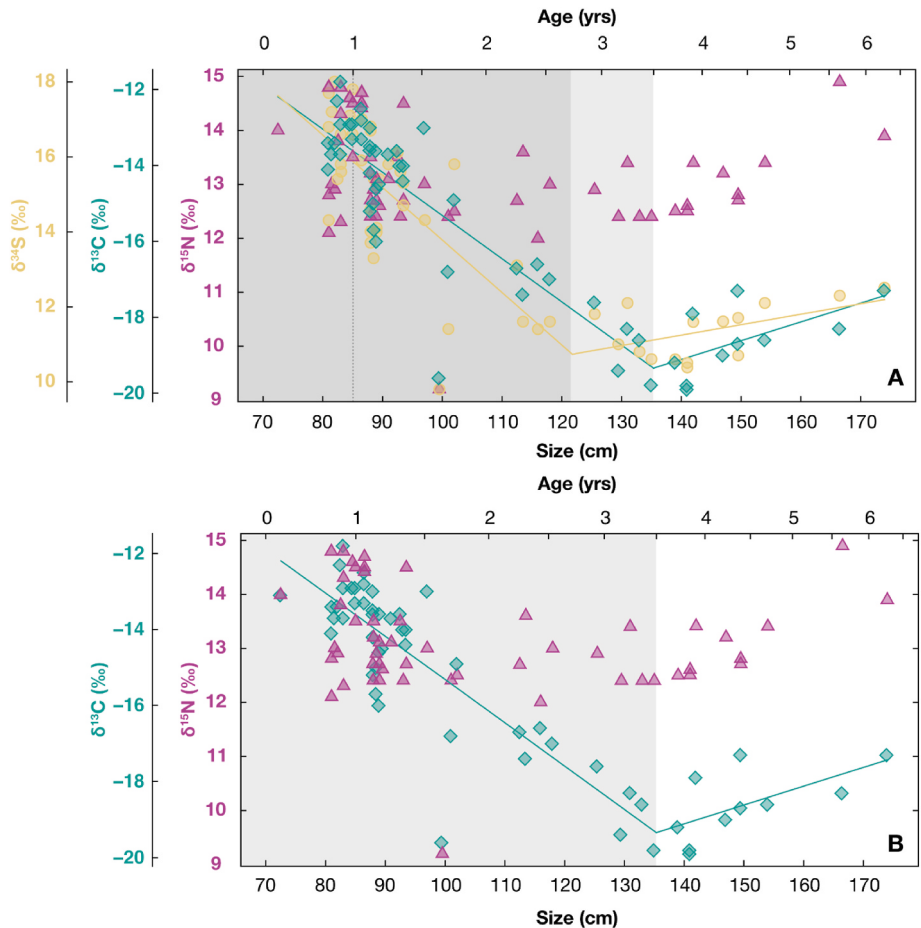


Fig. 2. Muscle stable isotope values of bull sharks of various lengths/ages, highlighting how incorporating S allowed us to confirm that the high young shark values < 3.5yrs/135 cm were driven by maternal influence rather than diet. Grey shaded area represents size classes that are influenced by maternal isotopes, as confirmed by the inclusion of sulfur. Figure readapted from Niella et al., (2021).

juvenile and young-of-the-year bull sharks from the Clarence River estuary (Australia) to assess diet. There was no significant relationship between $\delta^{15}\text{N}$ and size, suggesting no changes in trophic level with ontogeny (Fig. 2). However, changes were observed in $\delta^{13}\text{C}$ values, with individuals smaller than 135 cm (younger than 3.5 years) showing significant decreases whereas larger/older individuals had slight increases (Fig. 2). Young bull sharks are known to remain within their estuarine nursery systems for several years, prior to starting moving towards coastal and marine ecosystems as they grow (Werry et al., 2011). Higher $\delta^{13}\text{C}$ values in young-of-the-year and juvenile sharks observed (Fig. 2) suggest higher use of coastal regions than estuarine environments (Hussey et al., 2011), and therefore, are likely an artifact of maternal influence upon juvenile isotopic signatures. Nevertheless, using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values alone (as Fig. 2 demonstrates), in this instance, would make it difficult to confirm that maternal signatures are indeed influencing juvenile bull shark stable isotope values. By incorporating $\delta^{34}\text{S}$ values, Niella et al. (2021) were able to confirm the influence of maternal stable isotope values in young-of-the-year and juvenile bull shark isotope values, with values higher than what is plausible this far upstream in this estuary (>40 km), especially given the known progressive oceanic movement of this species with ontogeny, and the pelagic values measured in adults (Werry et al., 2011), and the known trend towards higher sulfur isotope values (21 ‰) near the ocean.

The segmented regression analysis used by Niella et al. (2021), showed that $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values in bull shark muscle tissues had similar inflexion points at around 3.5 years (or sharks larger than 135 cm). At this point, trends in juvenile bull shark isotopic values started to behave in accordance to what is known about the spatial ecology of this species, thus indicating the persistence of maternal effects for over 3 years post parturition, which is much longer than what was previously believed to be possible with muscle tissues, and especially so for rapidly growing juveniles. The exclusion of sulfur stable isotopes here would have led to inconclusive results, rather than those highlighting that tissue turnover in elasmobranchs is much longer than has been measured in captivity (usually under 1 yr as in Kim et al. (2012); Logan and Lutcavage (2010)).

4. Mixing models and diet

4.1. Sulfur isotopes and smaller discrimination factors

Assessing resource use (diet, habitat), the contribution of representative prey groups, or the contributions of different sources of primary production is perhaps the most common application of stable isotopes. Carbon and nitrogen stable isotope values are frequently used by themselves for these purposes. However, some of the key assumptions, or requirements, of mixing models suggest that including other tracers like sulfur would be beneficial. Sulfur stable isotope ratios, like carbon, have often been considered to show a relatively small amount of trophic discrimination (the trophic discrimination factor (TDF or $\Delta^{34}\text{S}$) between diet and the consumer's tissues following assimilation), especially compared to nitrogen. This has been identified as a major advantage when tracing the origin of material in food web studies where energy sources, or the prey that feed on them, differ considerably (e.g. across habitat gradients such as salinity, depth or between aquatic and terrestrial habitats). Although not nearly as well studied as for $\Delta^{13}\text{C}$ or $\Delta^{15}\text{N}$, the limited published summaries of $\Delta^{34}\text{S}$ values that exist (McCutchan Jr et al., 2003; Nehlich, 2015) indicate that values are low and close to zero. Indeed, in much of the literature there has been an assumption that $\Delta^{34}\text{S}$ is equal to zero, although tightly controlled feeding experiments by Florin et al. (2011) showed a strong ($R^2 = 0.89$) negative relationship between dietary $\delta^{34}\text{S}$ values and $\Delta^{34}\text{S}$ discrimination in rats and bears.

Here we have collected $\Delta^{34}\text{S}$ values based on controlled feeding experiments from the literature and summarised them by estimating mean ($\pm\text{SD}$) $\Delta^{34}\text{S}$ values from 164 different published estimates (see Table S1 for individual estimates). These individual estimates of $\Delta^{34}\text{S}$ vary with regard to the taxon under study (birds, fishes, invertebrates and mammals), the type of food fed to the consumer (plant, animal, mixed), the tissue analyzed (blood, brain, collagen, fur, hair, liver, muscle, whole organism) and to the length and type of feeding experiment. This is likely to lead to considerable noise in the data (cf. Florin et al., 2011) but reflects a marked increase in the availability of $\Delta^{34}\text{S}$ data compared to earlier summaries (McCutchan Jr et al., 2003; Nehlich, 2015).

Estimates of $\Delta^{34}\text{S}$ varied between -3.8 and 7.3 ‰ across the 164 different mean values we collected from the literature (Fig. 3). When all estimates were combined (all taxa, $n = 164$), the mean ($\pm\text{SD}$) $\Delta^{34}\text{S}$ was -0.4 (± 1.7) ‰. Although biologically similar to zero, this estimate was statistically different from zero (one-sample t -test: $t_{163} = -3.1$, $P = 0.002$). For birds ($n = 17$), mean empirical $\Delta^{34}\text{S}$ values from the literature varied between -3.8 and 0.6 ‰, with a mean $\Delta^{34}\text{S}$ of -1.0 (± 1.0) ‰, a value significantly different from zero ($t_{16} = -4.1$, $P = 0.008$). Fish ($n = 16$) $\Delta^{34}\text{S}$ values ranged between -1.1 and 4 ‰, and unlike the other taxa, fish showed a positive mean $\Delta^{34}\text{S}$ of 1.3 (± 1.3) ‰, which differed from zero ($t_{15} = 3.9$, $P = 0.002$). Estimates of $\Delta^{34}\text{S}$ from invertebrates ($n = 13$) showed the widest range (10.5 ‰) seen in all taxa (-3.2 to 7.3 ‰). The mean invertebrate $\Delta^{34}\text{S}$ value was 0.6 (± 0.3) ‰ and was not statistically different from zero ($t_{12} = 0.73$, $P = 0.50$). Mammals, the taxon with the largest number of controlled feeding experiments ($n = 118$) showed a considerable range of $\Delta^{34}\text{S}$ values (-3.7 to 4 ‰). The mean $\Delta^{34}\text{S}$ for mammals was -0.6 (± 1.3) ‰ and this was statistically distinct from zero ($t_{117} = -5.2$, $P < 0.001$) (see Fig. 3).

The ordinary least squares regression of values of empirically estimated $\Delta^{34}\text{S}$ on the $\delta^{34}\text{S}$ values of the food fed to the consumers in these experiments ($n = 107$) resulted in a noisy ($R^2 = 0.2$) but significant negative relationship (Figure 3) that differs considerably from that presented in Florin et al. (2011). Some authors have used Florin et al. (2011) to estimate $\Delta^{34}\text{S}$ from observed diet in field studies (Yohannes et al., 2014). Our results suggest that although there can be clear relationships between $\Delta^{34}\text{S}$ and diet $\delta^{34}\text{S}$ under tightly controlled conditions, this relationship cannot be applied in general, e.g., to other taxa or tissues.

Taken together, we have provided new estimates of $\Delta^{34}\text{S}$ that build on previous summaries (McCutchan Jr et al., 2003; Nehlich, 2015). Our results show that a small, but statistically significant amount of isotopic discrimination in sulfur does occur between consumption and assimilation ($\Delta^{34}\text{S}$) and that this varies not only between taxa (Fig. 3A) but also varies according to the $\delta^{34}\text{S}$ value of the food itself (Fig. 3B). Overall, the mean $\Delta^{34}\text{S}$ value across taxa was still notably lower than $\Delta^{13}\text{C}$ or $\Delta^{15}\text{N}$ (see Canseco et al., 2022), meaning in most cases the inclusion of $\delta^{34}\text{S}$ values for studies involving mixing models are less likely to be impacted by uncertainties associated with isotopic discrimination. These results underline the benefits of using probabilistic distributions from Bayesian statistics, and the need to focus on probabilities rather than absolute values of discrimination factors for mixing models.

We do not have the space here required to make a full examination of the factors driving these differences in $\Delta^{34}\text{S}$, but this is an area of future study that is of key interest to those using $\delta^{34}\text{S}$ values to understand the ecology and behavior of individuals, populations, and communities. The notable imbalance in the availability of $\Delta^{34}\text{S}$ estimates in the literature reveals a pressing need for more studies of $\Delta^{34}\text{S}$ in non-mammal taxa including birds, fish, and invertebrates.

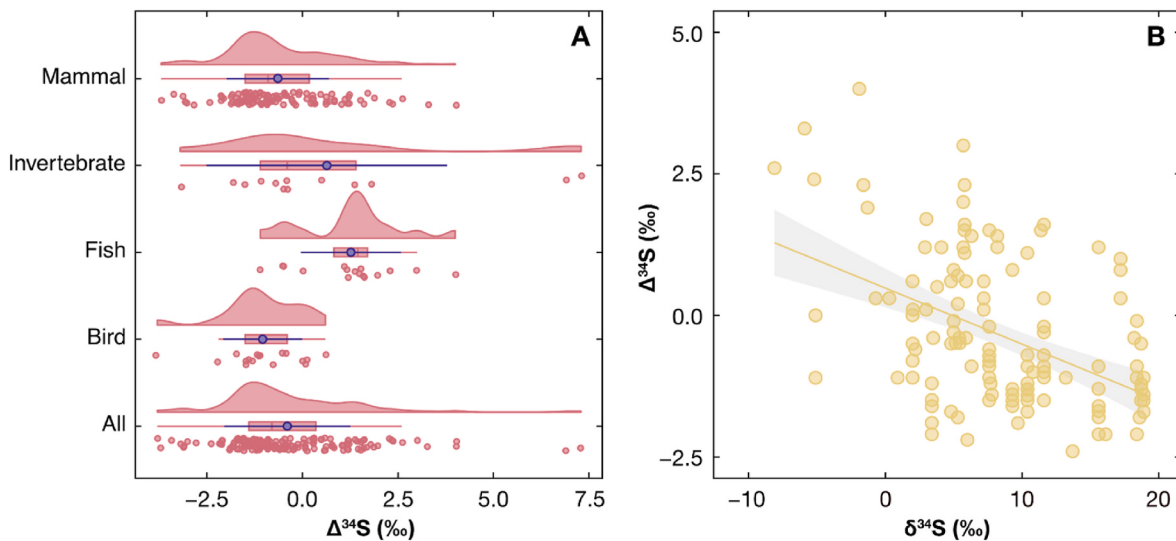


Fig. 3. A) Variation in estimates of $\Delta^{34}\text{S}$, the tissue-diet trophic discrimination factor (TDF) for sulfur estimated for different taxa including birds, fish, invertebrates, and mammals, and for all tax combined. Each marker relates to an individual empirical estimate from the literature for a certain feeding experiment of condition. Red circles and lines mark the estimated mean $\pm$ SD $\Delta^{34}\text{S}$ for a given taxon (values given in the main text), while the boxplots show the median $\pm$ IQR. A vertical line marks the commonly used zero value of $\Delta^{34}\text{S}$. B) Shows a noisy ($R^2 = 0.2$) but significant negative relationship between mean $\Delta^{34}\text{S}$ and the mean $\delta^{34}\text{S}$ value of experimental diet fed to consumers in each experiment. The regression equation is $\Delta^{34}\text{S} = 0.43 (\pm 0.19) - 0.11 (\pm 0.02) \times \delta^{34}\text{S}_{\text{diet}}$, $F_{1,140} = 36.3$, $R^2 = 0.20$, $P < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.2. Sulfur isotopes in mixing models

Smaller discrimination factors between consumer and prey can help lower uncertainty in mixing model outputs in addition to resolving more sources with the addition of a third tracer. The basic algebra of a linear mixing model mass balance model requires an additional tracer (ie. isotope) for each additional source. Even though the Bayesian mixing models typical used in modern ecology have allowed some flexibility in this context, the performance of stable isotope mixing models (e.g., *simmr*, *MixSIAR*) are typically improved where the optimal number of sources in a mixing model is $n + 1$, where n is the number of tracers in the model (Phillips et al., 2014). The inclusion of sulfur in addition to C and N isotopes therefore allows for more sources to be included in mixing models without negative impacts. The need for some degree of independence between the isotopes remains (Smith et al., 2013), though that may not always be the case if secondary effects lead to coupling of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Iii and Ferguson, 2012). Being able to incorporate more sources in mixing models is beneficial for multiple reasons:

Grouping of sources with overlapping isotopic compositions is a key step in mixing model preparation (Parnell et al., 2010; Stock et al., 2018). When analysing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values alone, it is highly likely that some sources overlap, since most biological tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are within the constrained range of -30 to -10 ‰ for $\delta^{13}\text{C}$, and 0 – 20 ‰ for $\delta^{15}\text{N}$. Having to group sources is often not ideal to answer ecological or biological questions because this reduces the resolution of outputs (fewer sources than ‘true’ available source). Mathematical grouping can even make results confusing when two overlapping sources with known different ecological roles have to be grouped. Including $\delta^{34}\text{S}$ values and/or other stable isotope ratios makes this overlap less likely because it adds another tracer with a broad range (-20 – 20 ‰) to the mixing model, which makes it more likely that sources will be isotopically distinct.

Another assumption of stable isotope mixing models is that all sources available to the consumer are in the model (Phillips et al., 2014). This naturally puts researchers in a complex situation, whereby there are a lot of biological sources that should be included, which results in sources more likely to overlap and invalidates the $n + 1$ source optimal

condition. Including another isotope such as sulfur in addition to carbon and nitrogen provides a little more leeway to avoid this conundrum. Together, these factors mean that the incorporation of $\delta^{34}\text{S}$ values can further refine mixing model outputs over a two-isotope approach (Pinzone et al., 2019; Pizzochero et al., 2018).

4.3. Sulfur isotopes and benthic/pelagic gradients

One of the drivers of fractionation in sulfur isotopes is bacterial fractionation in sediments (Fry et al., 1986; Matrosov et al., 1975; Mekhtiyeva et al., 1976), and as a result sulfur isotopes are useful for separating benthic and pelagic food webs (Fry et al., 2008; Peterson, 1999). Sulfur isotopes may be better at identifying pelagic gradients than $\delta^{13}\text{C}$ values which can be associated with benthic and pelagic patterns (Szpak and Buckley, 2020). For studies examining benthic-pelagic coupling, $\delta^{34}\text{S}$ values can discriminate between fish consumers feeding on benthic or pelagic sources (Cobain et al., 2024), and reduce credible intervals for assessments of pelagic contributions relative to $\delta^{13}\text{C}$ values alone (Duffill Telsnig et al., 2019).

Here we re-analyzed data from Burke et al. (2024) to demonstrate how $\delta^{34}\text{S}$ values can change the interpretation of studies of species occurring on benthic-pelagic gradients. Two species of sawshark with poorly understood trophic roles were examined using $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values, with fatty acid analysis in parallel. Various sources representative of pelagic, benthopelagic, and benthic sources were collected to assess the role of these species in connecting pelagic and benthic ecosystems. For both species, including sulfur isotopes in analyses resulted in greater contributions of pelagic sources, which represent greater than 50 % of contribution to diet in some cases, while not using sulfur increased the apparent contribution of benthic sources (Fig. 4). This significant contribution from pelagic sources was corroborated by fatty acid analysis, which suggested a strong contribution from pelagic dinoflagellates and cyanobacteria. Here, the inclusion of sulfur isotopes better represents pelagic contributions to aquatic food webs than using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values alone, and any studies where benthic-pelagic coupling is possible should consider their inclusion.

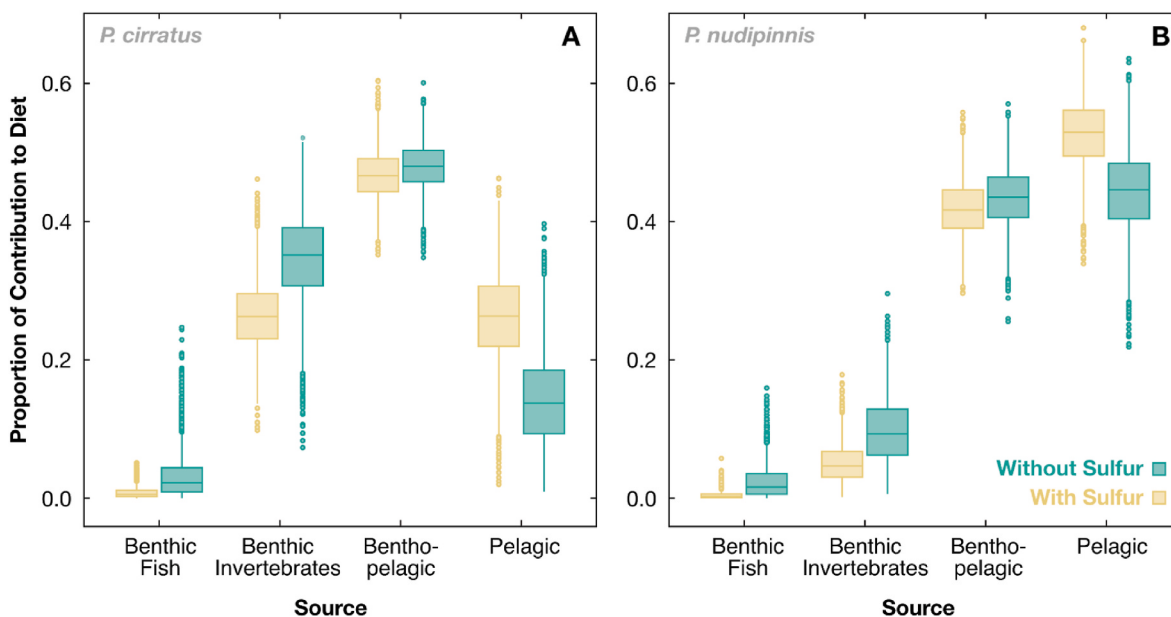


Fig. 4. Proportional contribution of different prey sources as modelled from Bayesian stable isotope mixing models of the common sawshark *Pristiophorus cirratus* (A) and the southern sawshark *Pristiophorus nudipinnis* (B) muscle tissue including sulfur (red) and excluding sulfur (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

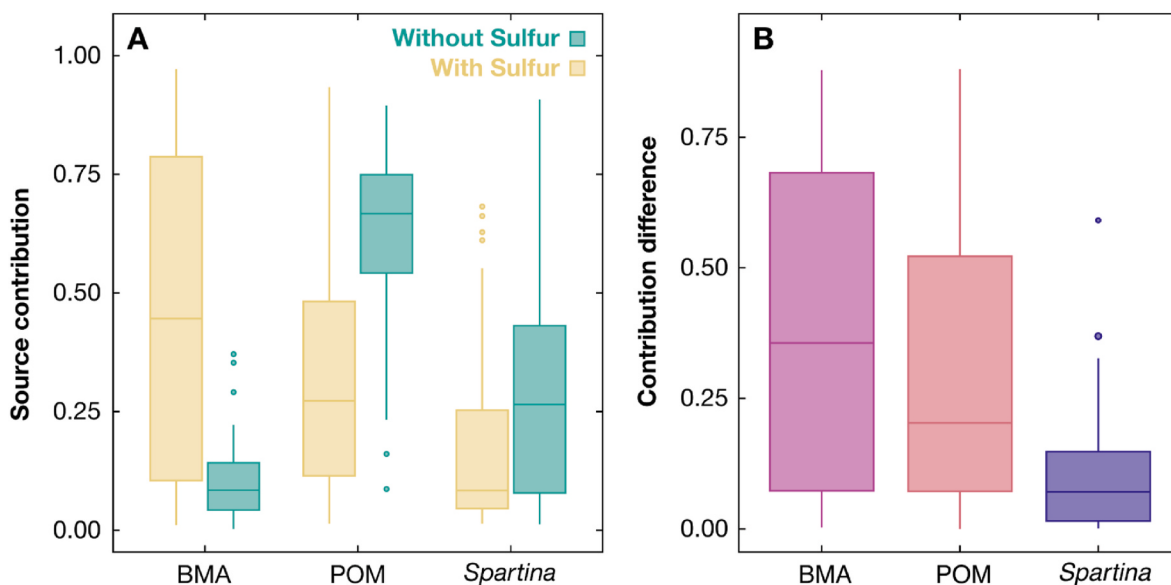


Fig. 5. Boxplot of the mean results for the Bayesian mixing model for all species across all years. Darker colors are outputs from mixing models that include sulfur and lighter colors are outputs of models that include only carbon and nitrogen. b) Boxplot of the difference in model outputs for each source across all species and years. BMA = benthic microalgae, POM = particulate organic matter. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.4. Mixing models to assess contributions of primary producers

Sulfur isotopes, when used with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, have a much greater likelihood of differentiating the contributions of primary producers in aquatic food webs (Connolly et al., 2004; Moncreiff and Sullivan, 2001). This is because primary producers have a wider range in sulfur isotope values compared to carbon and nitrogen (Connolly et al., 2004). Since bacterial processing affects sulfur isotope ratios, $\delta^{34}\text{S}$ values help differentiate benthic production from the rest of the food web, and can thus distinguish primary producer sources bound to sediment processes that have otherwise similar carbon pathways (and therefore similar $\delta^{13}\text{C}$ values (Mittermayr et al., 2014; Stribling and

Cornwell, 1997)). For example, without using sulfur isotopes, benthic organic matter appears to be a large contributor to the diets of fishes (Raoult et al., 2018), while the incorporation of sulfur isotopes finds little contribution by benthic production to the diets of fishes (Benstead et al., 2006; Heimbauer et al., 2024; Hewitt et al., 2020; Kharlamenko et al., 2001; Raoult et al., 2022b; Weinstein et al., 2000). The benefit of sulfur isotopes for assessing primary consumer contributions also extends to coastal environments, where researchers have identified the relative importance of macrophytic production (Kahma et al., 2020), and freshwater systems (Chanton and Lewis, 2002). Where researchers are interested in understanding how primary production flows through food webs, adding $\delta^{34}\text{S}$ values to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values will likely result in

more informative and accurate results.

To demonstrate how the incorporation, or exclusion, of $\delta^{34}\text{S}$ values can significantly change the outputs of studies examining contributions of primary producers, we re-ran Bayesian stable isotope mixing models using stable isotope data from seven consumer species collected from the Plum Island Longterm Ecological Research Site located in Rowley, Massachusetts in the northwest of the USA (<https://lternet.edu/site/plum-island-ecosystems-liter/>; (Deegan, 2021)). Collections of primary producers and consumers are made annually during the peak growing season from three locations along an estuarine gradient. Using the same species collected from the mid-estuary site we examined the effects of running mixing models with and without sulfur isotopes on samples collected over a seven year period (2009–2015) (Nelson et al., 2015). Mixing models were run for consumer species from each year using primary producers collected from that year (i.e. models for consumers collected in 2011 were run using primary producers collected in 2011). Each year, a mixing model was run using $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ or just $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values to compare the output of the two models and to understand the effect of using $\delta^{34}\text{S}$ values to estimate the source contribution of each primary producer to each consumer. Each model used three primary production sources (benthic microalgae (BMA), particulate organic matter (POM), and *Spartina*); therefore, each model was able to be explicitly solved mathematically.

In general, the mixing models using three isotopes estimated that all consumers were fuelled less by benthic microalgae and more by particulate organic matter and *Spartina* compared to the mixing models without $\delta^{34}\text{S}$ values (Fig. 5). The differences in model estimates when including $\delta^{34}\text{S}$ values were larger for benthic microalgae and particulate organic matter than *Spartina*. The relative source contributions varied by year with some being similar (e.g. 2013, Supplemental Table 1) while others had markedly different results (e.g. 2012, Supplemental Table 1), more accurately reflecting known production dynamics in Plum Island Sound. Additionally, the variation of source contribution estimates from mixing models that included sulfur were smaller than those that did not

(Supplemental Table 1). In this case the addition of sulfur isotopes improved both the resolution and confidence in the mixing model outputs.

5. Assessing movement

Stable isotopes are often used for identifying habitats of origin or seasonal movement for populations or individuals of interest (Hobson, 2019). This is done either with mixing models comparing stable isotope values of unknown populations with potential source populations/isoscapes, with complex statistical methods like Integrated Nested Laplace Approximation (INLA (St. John Glew et al., 2019)), or with more traditional statistical approaches (MacKenzie et al., 2011, 2014). The use of $\delta^{34}\text{S}$ values to assess movement has historically relied on using tissues with higher concentrations of sulfur, namely fur and feathers (Brlik et al., 2023; Hobson and Kardynal, 2016; Valenzuela et al., 2011). In these mainly terrestrial applications, $\delta^{34}\text{S}$ values can have large (0–20 ‰) spatial gradients which make them useful for spatial analyses (Date et al., 2022; Kabalika et al., 2020; Zazzo et al., 2011). $\delta^{34}\text{S}$ values are also independent from $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and thus more informative (Newton, 2021). Across terrestrial-marine gradients, marine aerosol transport produces a consistent gradient in $\delta^{34}\text{S}$ values detectable at local to continental scales (Bataille et al., 2021; Bern et al., 2015; Wadleigh and Blake, 1999). Thus, this terrestrial-marine gradient in $\delta^{34}\text{S}$ values can be used to assess movement in estuarine systems (Hesslein et al., 1991). Like the benefits of sulfur isotopes in mixing models, the addition of $\delta^{34}\text{S}$ values can also improve the resolution of movement analyses in marine ecosystems (Slessor and Trueman, 2021).

In aquatic systems, sulfur varies spatially in coastal ecosystems as a result of benthic-pelagic cycles and rates of freshwater input (St. John Glew et al., 2019). While carbon, hydrogen or oxygen isotopes may be more useful for large-scale movement studies (e.g., Raoult et al., 2020), sulfur isotopes are of particular use for movement applications in estuaries or freshwater systems, where there is a sulfur gradient between

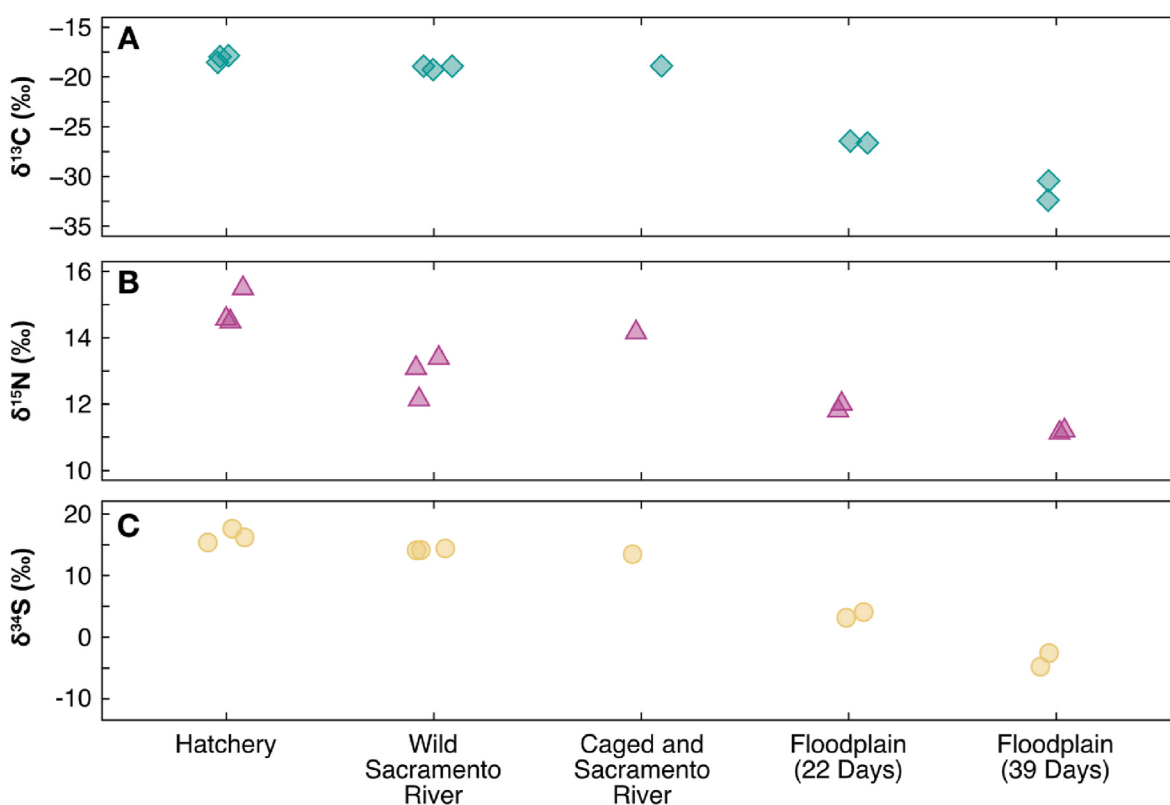


Fig. 6. Results from isotopic analysis from combined and homogenized laminae, excluding the core, highlighting the differences in habitats with each isotope.

Table 3

Results from Linear Discriminant Analysis (LDA) using various isotope combinations to determine the best predictor of habitat.

DFA Variables	Hatchery	River	Floodplain	Sum
Location ~ $\delta^{13}\text{C}$	0%	100%	100%	72%
Location ~ $\delta^{15}\text{N}$	100%	50%	100%	82%
Location ~ $\delta^{34}\text{S}$	33%	100%	100%	82%
Location ~ $\delta^{13}\text{C} + \delta^{15}\text{N}$	100%	75%	100%	91%
Location ~ $\delta^{13}\text{C} + \delta^{34}\text{S}$	67%	100%	100%	91%
Location ~ $\delta^{15}\text{N} + \delta^{34}\text{S}$	100%	75%	100%	91%
Location ~ $\delta^{13}\text{C} + \delta^{15}\text{N} + \delta^{34}\text{S}$	100%	75%	67%	82%

fully freshwater and fully marine systems (Fry and Chumchal, 2011).

To demonstrate how incorporating sulfur isotopes into movement studies can be beneficial, here we re-analyse data from Bell-Tilcock et al. (2021) with and without $\delta^{34}\text{S}$ values. Fish eye lenses were processed to obtain $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values, which were then used to identify which rearing habitats were used by juvenile Chinook salmon (*Oncorhynchus tshawytscha*). In the California Central Valley, Chinook salmon have three main freshwater rearing pathways depending on hydrologic conditions that occur i.e., the floodplain, river, or reared in a hatchery before migrating to the estuary and then the ocean (Bell-Tilcock et al., 2021; Goertler et al., 2018; Sommer et al., 2001). While in this example, the results appear to be the same using both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values (Fig. 6; Table 3), this result could be deceiving. In the California Central Valley, there is the potential to have overlapping $\delta^{13}\text{C}$ sources due to the detrital inputs in both freshwater floodplains and other more estuarine wetland habitats (Bell-Tilcock et al., 2021). Additionally, the invertebrate prey items for juvenile salmon in each of these habitats tend to occupy the same trophic level, which leads to overlapping $\delta^{15}\text{N}$ values (Bell-Tilcock et al., 2021). Whereas with $\delta^{34}\text{S}$ values, there is a gradient from freshwater-to-saltwater, with $\delta^{34}\text{S}$ values increasing as the salinity increases (Fry, 2002). This gradient spans ~25 ‰, whereas $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values vary by 15 ‰ and 4 ‰ respectively. This higher variation allows $\delta^{34}\text{S}$ values to be the main discriminator between freshwater floodplains from other wetland habitats in the

California Central Valley to identify the contributions of freshwater floodplains as rearing habitats.

In addition, for identifying floodplain contribution, $\delta^{34}\text{S}$ values are the main identifier used to differentiate between hatchery and naturally spawned Chinook salmon. In the California Central Valley, only a fraction of the fish reared in hatcheries are marked with a fin clip and a coded wire tag (Satterthwaite et al., 2015). This can make investigations linking environmental conditions in the freshwater to the success of progeny from natural spawning salmon difficult to study due to the large amount of unmarked hatchery fish in the system. In the hatcheries young salmon are fed a diet of marine based protein, resulting in them having marine-like $\delta^{34}\text{S}$ values (Hurd et al., 2008). However, $\delta^{15}\text{N}$ values can vary across the landscape due to various inputs throughout the system (Bell-Tilcock et al., 2021 supp material), reducing the utility of $\delta^{15}\text{N}$ values to discriminate hatchery origin salmon. In our example below, the high $\delta^{34}\text{S}$ values were permanently recorded in fish eye lenses. Having high $\delta^{34}\text{S}$ values in their lenses during their early life history, gives us a tool to differentiate hatchery origin salmon that can be used for more accurate reconstructions of growth, survival, and life history diversity of naturally spawned salmon.

6. Oceanography

To better constrain sources, sinks, and various dynamics within marine nutrient cycles, oceanographers have studied $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in various organic matter pools for over six decades (Beaupré, 2015). Most studies measure bulk pools, like dissolved (DOM) or particulate organic matter (POM; Benner et al., 1997), but increasingly $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are measured in individual compounds like amino acids (Ruiz-Cooley et al., 2014; Stahl et al., 2023). Sulfur, despite being a large and critical component of all of these organic pools (Ksionzek et al., 2016), has not been a priority in oceanographic isotope studies (Mekhtiyeva et al., 1976; Sweeney et al., 1980).

In this example, we explore the potential utility of adding $\delta^{34}\text{S}$ values to ongoing oceanographic isotope studies of organic matter, namely DOM (Fig. 7). Measurements of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are common for

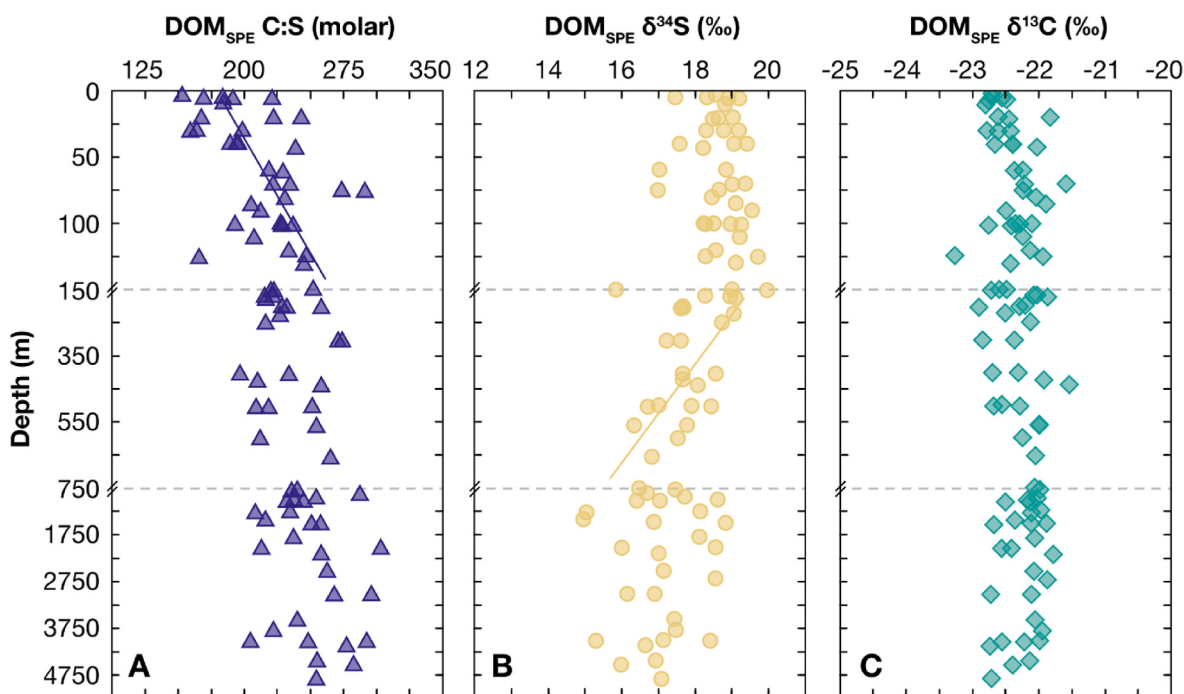


Fig. 7. Adding sulfur to marine dissolved organic matter (DOM). Oceanographers often use stable isotope ratios to elucidate sources to marine DOM. However, the narrow range of $\delta^{13}\text{C}$ values limits utility. Adding sulfur to measurements allows two new variables, molar C:S ratios and $\delta^{34}\text{S}$ values, which have a greater dynamic range and produced significantly significant trends at different depth horizons.

DOM, so much so that they are routinely measured as part of the NSF-led Hawaii Ocean Time-series (HOT) and Bermuda Atlantic Time Series (BATS). Yet, prior to 2022, no $\delta^{34}\text{S}$ values of marine DOM had been reported. Using the same method for isolation and characterization of DOC and DON (Dittmar et al., 2008), Phillips et al. (2022) measured 100 samples for $\delta^{34}\text{S}$ values across ocean basins, including at HOT and BATS. There were two immediate benefits from this addition of sulfur. First, the $\delta^{34}\text{S}$ values in marine DOM had more than double the dynamic range ($\sim 5\text{‰}$) than $\delta^{13}\text{C}$ values ($\sim 2\text{‰}$), similar to common ranges in $\delta^{15}\text{N}$ values ($\sim 8\text{‰}$). Second, adding sulfur to the EA-IRMS analysis allowed simultaneous determination of molar C:S ratios (complementing typical C:N ratios). These additional constraints allowed the authors to disprove a leading hypothesis on sources to DOM that were unresolvable from C and N alone. For future oceanographic studies, especially existing longer term time-series, we recommend the expansion beyond C and N alone, adding S to DOM and POM workflows.

7. Bioarchaeology

Many of the issues discussed in previous sections related to improved interpretations related to diet, habitat use, and movement in contemporary ecosystems apply well in archaeological and palaeontological contexts. In paleontological fields, the preservation of sulfur isotopes in mineralized structures, like dorsal spines (Luccisano et al., 2023) or bones (Goedert et al., 2018), allows answering of questions relating to the use of euryhaline movements and palaeoecological reconstructions. Sulfur isotopes have also been used to study early (3.4 billion years ago) biogeochemical processes in microbial mats (Bontognali et al., 2012) and euxinic oceanic patterns that may have contributed to mass extinctions (Sim et al., 2015; Thompson and Kah, 2012). Observations of mass independent fractionation in sulfur isotopes have also provided some of the strongest evidence for the Great Oxygenation Event (Farquhar and Wing, 2003). Despite its potential use for palaeoecological questions, sulfur stable isotopes have mostly been used in archaeological contexts.

Most applications of stable isotopes in archaeological contexts are focused on humans, especially their diets and mobility patterns. Sulfur isotope compositions are determined primarily from collagen extracted from ossified tissues (bones and teeth (Nehlich, 2015)) and less commonly hair where organic preservation is outstanding (Britton et al., 2018; Fernández et al., 1999; Hyland et al., 2021; Wilson et al., 2007). With respect to human mobility, sulfur isotopes offer a particularly attractive and relatively unexplored frontier as there is often geographic variation in terrestrial $\delta^{34}\text{S}$ values according to proximity to the coast (Zazzo et al., 2011) or underlying geology (Ebert et al., 2021; Kabalika et al., 2020). Traditionally, mobility is explored using strontium isotope measurements of tooth enamel, which forms early in life and does not

remodel (Bentley, 2006). Bone, which continuously remodels and reflects a weighted average of dietary intake over many years before death (Hedges et al., 2007), does not produce reliable strontium isotope ratios because of high levels of porosity and the small size of bioapatite crystals in bone (Hoppe et al., 2003). Therefore, studies of human mobility using strontium isotopes provide insight into geographic location during the first ~ 12 years of life, regardless of the individual's age at death (Fig. 8). $\delta^{34}\text{S}$ values of collagen extracted from dentine provide a longer time series, particularly if serially-sampled (Eerkens et al., 2011), extending into adulthood. There has been a great deal of interest surrounding various methodological applications geared towards incremental sampling of tooth dentine for isotopic analysis, but only recently has sulfur isotope measurements been considered (Cheung et al., 2022). Moreover, because sulfur isotope compositions can be determined for bone collagen, the possibility of determining the most recent sulfur isotope composition exists whereas it does not for strontium (Fig. 8). Sampling multiple bones with different turnover rates offers another possible means of resolving diachronic variation in adult diet (Cox and Sealy, 1997), but currently our knowledge of differences in collagen turnover rates among skeletal elements is highly qualitative (Fahy et al., 2017).

The extraction of aquatic resources by human populations has been an important aspect of subsistence for millennia (Erlandson, 2001). Humans have primarily relied on coastal marine fauna and sulfur isotope analysis offers some distinct advantages with respect to understanding how humans interacted with these species and marine environments more generally. Guiry et al. (2021) analyzed $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values of sheephead (*Archosargus probatocephalus*) from archaeological sites in the Gulf of Mexico dating from 2500 BP to 1890 CE. They found that the size of sheephead landed decreased between the 1720s and 1830s and there was an expansion of fishing into more distant seagrass meadows on the basis of high $\delta^{13}\text{C}$ and low $\delta^{34}\text{S}$ values in sheephead postdating 1820 CE. This shift in fishing practices was clearly resolved by the addition of the $\delta^{34}\text{S}$ values as the $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values alone provided ambiguous results. The addition of a novel isotope tracer is crucial in addressing the equifinality in interpretations that often characterizes studies relying on stable isotopes, particularly in archaeological contexts when dietary sources and the base of the food web are rarely, if ever, adequately sampled. Given the variation in the $\delta^{34}\text{S}$ values that exist in aquatic organisms among different habitat types (Barros et al., 2010; Szpak and Buckley, 2020; Yamanaka et al., 2000), there is great potential for applying similar methods to better understand where and how past human populations were exploiting aquatic environments.

The importance of aquatic resources during the Mesolithic Period and the Early Neolithic in Europe have also received considerable attention. The consumption of freshwater fish has been discussed extensively in this context and isotopic data have played a major role in

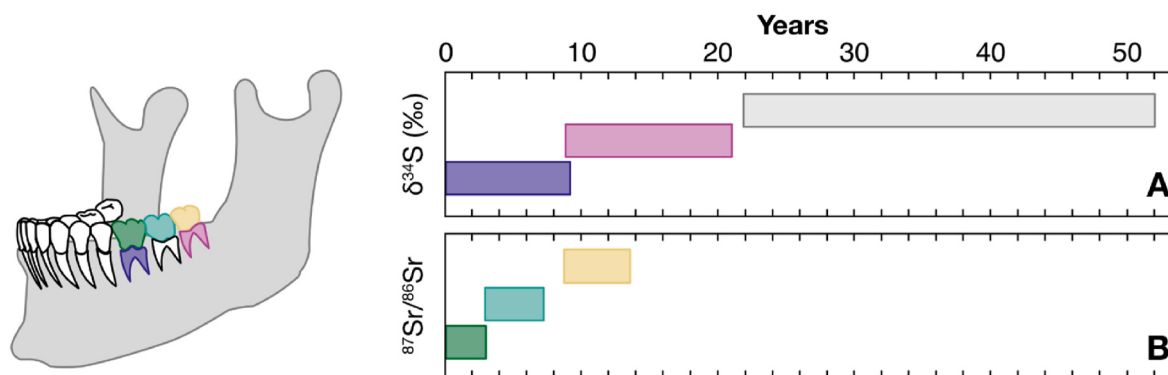


Fig. 8. Sulfur isotope compositions of bone and tooth collagen provide an opportunity to study diachronic variation in the mobility of past human populations that would not be possible using strontium isotope systems. The shapes at the top of the figure correspond to different tissues and their relative formation or remodelling times. The cross hatching on the teeth indicates the diachronic resolution that is afforded by sequential sampling of these tissues.

testing hypotheses about human diet in these periods. Specifically because most European sites, especially in central and northern Europe, are devoid of C4 plants, there tends to be little variation in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in potential plant and animal foods, as well as the humans themselves. Sulfur isotopes have been particularly attractive in this context as there may be large differences in the $\delta^{34}\text{S}$ values of freshwater and terrestrial resources. Bollongino et al. (2013) used $\delta^{34}\text{S}$ values of bone collagen to clearly differentiate genetically distinct Neolithic foragers from Neolithic hunter-gatherers from Blätterhöhle cave in central Europe. Their interpretations were, however, based on a single $\delta^{34}\text{S}$ value from a fish bone that could not be identified to a specific taxon. Similarly, Nehlich et al. (2010) interpreted differences in $\delta^{34}\text{S}$ values between Mesolithic and Neolithic human groups from the Danube Gorges region of southeastern Europe as evidence for variable reliance on freshwater fish. As with the previous study, a very small number of $\delta^{34}\text{S}$ values ($n = 3$) were used to characterize the aquatic resources. The greater capacity to determine $\delta^{34}\text{S}$ of collagen, with its very low sulfur content, accurately and precisely with newer instrumentation with certainly improve the capacity of such studies to interpret the relative importance of freshwater resources with less ambiguity caused by insufficiently characterizing source isotopic compositions. Recently, two studies have extensively sampled archaeological fauna from different habitats and urge the consideration of $\delta^{34}\text{S}$ values to better understand how past human populations used wetland habitats, whether through the direct collection of faunal resources or via the grazing of livestock in these environments (Guiry et al., 2022; Lamb et al., 2023). Future studies that better address and incorporate the biogeochemical processes underlying variation in $\delta^{34}\text{S}$ across different types of environments will likely provide useful avenues for research into how ancient human populations interacted with the landscape.

8. Discussion and strengthening use of sulfur isotopes

Given the broad range of use cases presented above and increasing feasibility of conducting sulfur stable isotope analysis, it is clear that more researchers should consider the addition of $\delta^{34}\text{S}$ values to their studies. However, there remains a substantial amount of work to do before sulfur isotopes fully become a standard ecological tool:

One of the key requirements of using stable isotope tracers for ecological applications is a good understanding of diet-tissue discrimination factors, trophic enrichment, and any interactive effects of inorganic content (Shipley and Matich, 2020). For C and N stable isotopes, there are now wide-scale meta-analyses available to inform ecological studies using these tracers, e.g. Canseco et al. (2022), which not only gives greater confidence in model outputs, but also a better understanding of the uncertainties therein. The preliminary meta-analysis presented in this review demonstrated that while mean $\Delta^{34}\text{S}$ values are lower than for other isotopes, there is a considerable variability in these values across and within taxa. The negative relationship between $\Delta^{34}\text{S}$ and diet $\delta^{34}\text{S}$ values is also different to other isotopes and warrants further explanation. Clearly, more targeted studies examining not only the patterns in $\Delta^{34}\text{S}$ values across different taxa but also causal explanations for their variation are required.

Studies examining effects of chemical pretreatments on sulfur stable isotopes are still very sparse. Lipid extractions can have large (>5 ‰) effects on $\delta^{34}\text{S}$ values in some tissues like liver (Riverón et al., 2022), possibly due to removal of sulfolipids with differing $\delta^{34}\text{S}$ values to the majority of the source tissue. For muscle tissues, lipid extraction does not seem to have significant effects, but few species have been explicitly tested ((Larocque et al., 2021) salmonids; (Javornik et al., 2019), bears). Preservation methods that often include ethanol or formaldehyde can also have effects on isotopic values, but do not seem to impact $\delta^{34}\text{S}$ values (Javornik et al., 2019; ethanol) because KIE is not expressed in the S atom. Further, protein separation of sulfur containing amino acids, especially cysteine may result in isotope fractionation if sulfur is not protected - with a 15 ‰ normal kinetic isotope effect observed during

oxidation to cysteic acid (Phillips et al., 2021). Acidification of samples, a common treatment for sediments or organic material that contains inorganic minerals, can also change sulfur stable isotope values relative to untreated samples by up to 7 ‰ (Connolly and Schlacher, 2013). Studies that explicitly test for the effects of chemical treatment on sulfur stable isotopes are necessary to correct for the likely effects.

Carbon and nitrogen stable isotope measurements have clear, internationally-agreed reference samples that allow comparison of results between studies: VPDB and atmospheric nitrogen (Coplen et al., 1983; Gröning, 2004). Reference values for $\delta^{34}\text{S}$, however, have been adjusted repeatedly (Mann et al., 2009), and there are numerous internationally-approved reference materials (e.g. IAEA-S-1, 2, 3) that may be used in parallel or independently. This has created issues comparing biologically-sourced samples (Zhao and Zhao, 2021), and results in different analytical laboratories using different inorganic standards. This could create uncertainty when comparing studies that may have used different reference materials, and further highlights that researchers need to be clear in reporting which reference materials were used (Skrzypek et al., 2022). These issues are likely to be exacerbated as instrumentation such as MC-ICP-MS and Orbitrap MS grow in popularity, as the format of existing standards need to be adapted to this new instrumentation and correctly calibrated (Paris et al., 2013; Silverman et al., 2022). Cross-laboratory studies that explicitly assess these uncertainties like those conducted for other isotopes (Orlowski et al., 2018; Pestle et al., 2014) would help address this issue more broadly to allow direct cross-study comparisons.

Precision of sulfur isotope measurements (± 0.5 ‰) are typically lower than that of carbon and nitrogen (± 0.1 ‰, see Raoult et al. (2019)), and despite these variations being less than ecologically-meaningful effects, they could lead potential users of sulfur to avoid its use. More sensitive detectors are becoming more widespread - advancements in separation of SO_2 from mixture of combusted gases. EA-IRMS is currently the most robust approach for simultaneous measurements of C, N, and S isotopes at low cost. Future decades may see even more analytical advancements with GC-MC-ICPMS or OrbitrapMS, but these still need substantial method development before broad adoption by laboratories.

A unique concern for stable isotope measurements of biological samples of ancient origin is the possibility that the isotopic composition has been altered in the burial environment. A fairly large body of literature exists that explores how various quality control measures can be used to ensure the reliability of stable carbon and nitrogen isotope measurements of bone collagen (reviewed in Guiry and Szpak (2021). Nehlich and Richards (2009) proposed some quality control measures for $\delta^{34}\text{S}$ value measurements of ancient collagen based on sulfur elemental compositions, but there is no evidence that these criteria correlated with more or less reliable $\delta^{34}\text{S}$ values. There is, therefore, still uncertainty with respect to ensuring the authenticity of $\delta^{34}\text{S}$ data generated from ancient samples and addressing this gap should be a priority area for future research.

Some of the more logistical barriers to the broader use of sulfur isotopes in aquatic sciences are the perceived higher analytical costs, as well as the difficulties associated with storing large quantities of SO_2 required for sulfur analysis. Historically, the difficulties associated with processing samples to measure $\delta^{34}\text{S}$ values meant that analytical costs, in conjunction with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, approximately doubled total cost per sample (for example, UC Davis, one of the World's most active service providers, currently charges \$9 USD for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, but the price increases to \$34.5 USD with $\delta^{34}\text{S}$ values). Improvements in analytical pipelines and increasing interest in the analysis of this isotope mean that current costs for determining $\delta^{34}\text{S}$ values are moving towards parity with that of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, or increasing the total cost of analysing the three isotopes by approximately 30%. In some cases, the difference in analysing $\delta^{34}\text{S}$ in addition to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values is lower than parity: for example, at HK University, analysing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values comes at a cost of \$70 HKD per sample, whereas adding $\delta^{34}\text{S}$

values to this process adds just \$15 HKD, or 21% to the total cost (https://www.biosch.hku.hk/si_lab). Another barrier to broader use of sulfur isotopes is the difficulties in safely storing SO₂ gases that are used for analysis. Since SO₂ reacts with water to form sulfuric acid, it poses a threat to the environment, corrodes nearby metal components, and is a hazardous material for human health. While these threats are surmountable, institutions may be reluctant to allow the storage of this gas for analytical purposes. Highlighting the clear advantages that sulfur isotope analysis can bring to aquatic science is necessary for institutions to balance the risk of housing SO₂ with the benefits.

9. Conclusions

At worst, including $\delta^{34}\text{S}$ values in analyses will give greater confidence in the results that would have been provided by $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ alone. In many cases, as our examples demonstrate, using $\delta^{34}\text{S}$ values in addition to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ can lead researchers to completely new insights into various aspects of biological systems. The previous barriers to the inclusion of $\delta^{34}\text{S}$ values into studies, namely additional cost and greater tissue mass requirements, are progressively disappearing, with many detectors requiring <1.5 mg of tissue for animals or plants (similar to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). The analysis of $\delta^{34}\text{S}$ values are also able to be conducted in parallel with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values at ~30% greater cost. Our collective experiences with incorporating $\delta^{34}\text{S}$ values into studies using stable isotopes leads us to strongly argue for moving to sulfur, carbon and nitrogen isotopes as the 'baseline' for stable isotope research in aquatic systems.

CRediT authorship contribution statement

Vincent Raoult: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Alexandra A. Phillips:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **James Nelson:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Yuri Niella:** Writing – review & editing, Writing – original draft, Formal analysis. **Christina Skinner:** Writing – review & editing, Writing – original draft, Formal analysis. **Miranda Bell Tilcock:** Writing – review & editing, Writing – original draft, Formal analysis. **Patrick J. Burke:** Writing – review & editing, Writing – original draft, Formal analysis. **Paul Szpak:** Writing – review & editing, Writing – original draft, Formal analysis. **W. Ryan James:** Writing – review & editing, Writing – original draft, Formal analysis. **Chris Harrod:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data used in this paper are available and from already published sources

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Appendix A. Supplementary data

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