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FEATURE ARTICLE

Sample acidification significantly alters stable isotope ratios of sulfur in aquatic plants and animals

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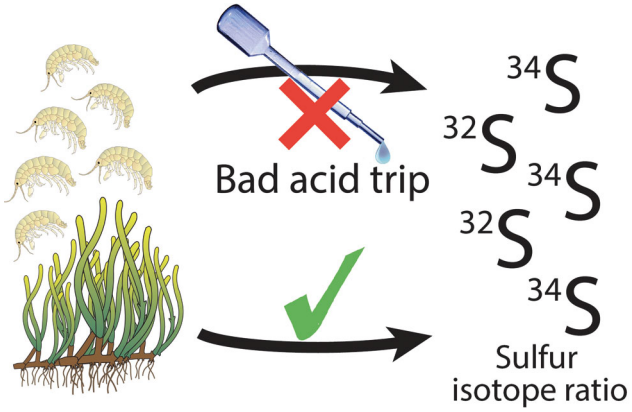
ABSTRACT: Sulfur stable isotopes are increasingly being used as tracers of material processing in studies of both modern and historical food webs. Preparation of plant and animal material for isotope analysis routinely includes steps that remove inorganic material not normally assimilated by consumers. Whereas acidification of samples is known to assist with this for some elements (carbon), it can produce unwanted effects for others (nitrogen). Here we tested the effects of acidification on sulfur isotopes by comparing isotope ratios of paired acidified and non-acidified samples of seagrass, epiphytic algae growing on seagrass and animal consumers (3 types of crustaceans: amphipods, copepods and isopods). Acid treatment resulted in significant losses of elemental sulfur from the tissues and changes in sulfur isotope ratios of samples. The artificial depletion of the heavy sulfur isotope decreased sulfur isotope ratios by 2.6‰ on average, and by as much as 7.0‰ in individual samples. Acidification of samples prior to sulfur isotope analysis results in invalid ratios and should not be used.

KEY WORDS: Acid digestion · Amino acids · Carbonate · DMSP · Seagrass · Stable isotope analysis



Acidification of samples prior to sulfur isotope analysis results in invalid ratios and should not be used.

Image: R. Connolly, Symbols: http://ian.umces.edu/symbols

INTRODUCTION

Over the last 2 decades, the use of stable isotope (SI) analysis of the light elements hydrogen, carbon, nitrogen, oxygen and sulfur has developed into a near-universal tool in ecology, with applications encompassing fields of investigations and disciplines spanning the length and breadth of ecology. Within the broader discipline of trophic ecology (sensu lato), SIs are commonly used to address 5 inter-related objectives: (1) to describe the structure of food webs and to identify the principal energetic pathways that make up food webs (Olson et al. 2010, Hilting et al. 2013), (2) to identify the main food categories of consumers (Winning et al. 1999, Oakes et al. 2010a, Le Pape et al. 2013, Vander Zanden et al. 2013), (3) to assess the probable inputs from different source materials to the diet of consumers (Melville & Connolly 2005, Oakes et al. 2010b, Hyndes et al. 2013), (4) to estimate the length of food chains and trophic levels of consumer species (Post 2002, Layman et al. 2012) and (5) to reconstruct historical diets (Froehle et al. 2012).

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Movement and transformations of organic matter are key processes in ecosystems and food webs, and SI techniques are widely employed in this context to determine the provenance of organic matter and its subsequent biogeochemical transformations in detrital and sediment pools (Schlacher & Wooldridge 1996, Connolly et al. 2005a, Rosenbauer et al. 2009). SI analysis is also employed to follow the exchanges of organic matter and nutrients between ecosystems and habitats (Gaston et al. 2006, Connolly et al. 2009, Fry 2011, Piovia-Scott et al. 2013), and to track animal movements (Rubenstein & Hobson 2004). In pollution studies, SIs are effective chemical tracers to identify pollutant inputs and the movement of toxicants through food webs (Schlacher et al. 2007, Connolly et al. 2013, Jardine et al. 2013).

Naturally occurring SI ratios of S can be very useful as tracers of the fate of assimilated nutrients in aquatic food webs, particularly when the more routinely used elements, C and N, cannot resolve food web questions (Peterson & Howarth 1987, Connolly et al. 2004). The relatively high variability of S isotope ratios over small spatial scales can help determine physiological and ecological processes in aquatic systems (Oakes & Connolly 2004). In food web studies, S is particularly effective as a complementary element to C for source determination (Connolly et al. 2004)

Preparation of aquatic plants and animals for mass spectrometry can involve acidification of the samples (Table 1). Acid treatment is done to remove inorganic carbonate (contained in calcified biological struc-

Table 1. Examples of variability in sample preparation for sulfur stable isotope measurements of biological material used in food web studies with respect to acid treatment prior to mass spectrometry. The studies listed are illustrative and are not intended to cover the full range of the literature on $\delta^{34}S$ in aquatic plant and animal samples

	Samples acidified	No acid treatment
Primary producers		
Benthic microalgae	Weinstein et al. (2000), Howe & Simenstad (2011)	Wainright et al. (2000), Wilson et al. (2009)
Phytoplankton	Moncreiff & Sullivan (2001), Howe & Simenstad (2011)	Wainright et al. (2000), Maier et al. (2011)
Macroalgae (e.g. *Catenella, Cladophora, Dictyota, Enteromorpha, Gracilaria, Rhizoclonum, Ulva*)	Currin et al. (2011), Howe & Simenstad (2011)	Granek et al. (2009), Wilson et al. (2009)
Marsh plants (e.g. *Aster, Distichlis, Juncus, Phragmites, Salicornia, Scirpus, Spartina, Typha*)	Attrill et al. (2009), Howe & Simenstad (2011)	Wozniak et al. (2006), Attrill et al. (2009)
Seagrass (e.g. *Halodule, Halophila, Posidonia, Ruppia, Syringodium, Thalassia, Zostera*)	Oakes & Connolly (2004), Hindell & Warry (2010), Currin et al. (2011)	Holmer et al. (2009), Wilson et al. (2009)
Mangroves (*Avicennia, Bruguiera, Rhizophora, Sonneratia*)	Newell et al. (1995), Hindell & Warry (2010)	Benstead et al. (2006), Granek et al. (2009)
Consumers		
Zooplankton	Moncreiff & Sullivan (2001), Howe & Simenstad (2011)	Rissik et al. (2009), Belicka et al. (2012)
Sponges & Cnidaria (corals)	Becker et al. (2009)	Granek et al. (2009)
Peracarid crustaceans (e.g. amphipods, isopods, mysids, cumaceans)	Becker et al. (2009), Howe & Simenstad (2011)	MacAvoy et al. (2002)
Decapod crustaceans (e.g. crabs, prawns, shrimp, squat lobsters)	Becker et al. (2009), Howe & Simenstad (2011)	Leakey et al. (2008), Wilson et al. (2009)
'Worms' (e.g. polychaetes, oligochaetes, flat worms, sipunculids)	Newell et al. (1995), Becker et al. (2009)	MacAvoy et al.(2002), Wilson et al. (2009)
Molluscs (e.g. gastropods, bivalves, squid)	Howe & Simenstad (2011), Belicka et al. (2012)	Granek et al. (2009), Wilson et al. (2009)
Echinoderms (e.g. sea urchins, brittle stars, sea cucumbers)	Newell et al. (1995), Becker et al. (2009)	Vaslet et al. (2012)
Fish	Stribling & Cornwell (1997), Howe & Simenstad (2011)	Belicka et al. (2012)
Birds	Kwak & Zedler (1997)	Moreno et al. (2011)
Marine mammals	Barros et al. (2010)	Craig et al. (2006)

tures, such as shells and skeletons, or in sediments suspected to contaminate the samples) that would bias the isotope signal ($\delta^{13}C$) of organic matter that is of primary interest in food web studies. Treating samples with acids to remove carbonates can, however, have unintended consequences for nitrogen isotopes ($\delta^{15}N$) that are altered during acid treatment (Bunn et al. 1995). This artefact introduced by acid treatment on $\delta^{15}N$ is variable and generally not predictable (Bosley & Wainright 1999, Kennedy et al. 2005).

Sulfur isotopes have, historically, been more difficult and expensive to analyse than the routinely measured elements C and N. The application of S isotopes in food web studies has increased markedly in recent years, a trend that tracks the wider adoption of isotope techniques in ecology (Fig. 1). However, there is no consistency among investigators with regards to acidification of sample material prior to the analytical determination of S isotope ratios ($\delta^{34}S$), and no standard procedures exist regarding whether or not to acidify different types of biological materials (Table 1).

Acid treatment causes a bias in isotope analysis of N, which occurs predominantly in proteins. Most organic S is also found in proteins, so acid treatment might also affect S ratios. With the increasing use of S isotopes, there are widespread inconsistencies in sample treatment (as seen in Table 1), making it important to know the effects of acidification on S isotopes. At present, no report has investigated any such possible effects. Here we determined acid-treatment effects on S isotope ratios in biological material representing key producers and consumers of an aquatic food web.

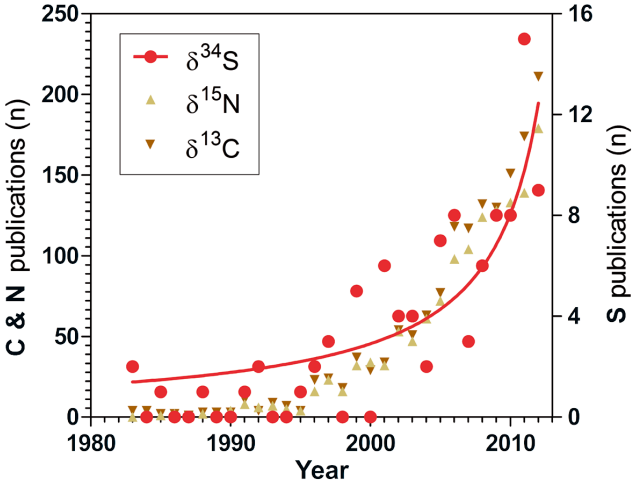

Fig. 1. Trend in the number of papers that used stable isotopes of sulfur, carbon or nitrogen in studies of food webs or trophic processes. Source: www.scopus.com. Search terms: ((*## AND isotope**) *OR* (*$ AND isotope**)) *AND* (*foodweb** *OR food web** *OR trophic*), where ## = sulfur, or nitrogen, or carbon, and $ = sulphur

MATERIALS AND METHODS

Plants, algae and small animals associated with seagrass meadows were sampled on the east coast of the Gulf St Vincent in South Australia (34° 00′ S, 138° 00′ E), at sites previously used by Connolly et al. (2005b). We collected numerous samples of each organism type at multiple locations as part of a larger food web study, but for testing the effects of acidification, we selected the 3 samples from locations that provided the greatest quantity of material (especially important for samples of algae and small animal consumers). We used 3 species of seagrass from shallow coastal waters (*Posidonia australis*, *P. sinuosa*, *Zostera muelleri*). Each seagrass sample consisted of 3 leaves from different shoots, scraped clean of conspicuous epiphytic algae with a razor blade and rinsed in distilled water (see Guest et al. 2004). For algae samples, we used epiphytic macroalgae removed from 2 of the seagrass species, *P. australis* and *Z. muelleri*. These were short, fine filaments comprising a mixture of green, red and brown algae. We used 3 types of crustaceans, collected from the seagrass canopy using fine-mesh sweep nets: gammarid amphipods (multiple species), harpacticoid copepods (multiple species) and isopods (*Platynympha longicaudata*). Samples consisted of numerous individual animals (amphipods >20, copepods >100, isopods >5). For each of these 8 organism types, we had 3 replicate samples. Sample material was dried at 60°C to constant weight, ground to a fine powder using mortar and pestle and split into 2 aliquots (both >5 mg), one for analysis without any further processing, the other for analysis after acid treatment.

The procedure for acid treatment consisted of slowly adding drops of HCl (1 M) to dry, powdered material in glass vials until all gas production had ceased. We elected not to further rinse the acidified sample with distilled water before re-drying since it has been shown that, for C isotopes, rinsing can in itself affect isotope ratios (Mateo et al. 2008).

The ^{34}S:^{32}S ratio was calculated as the relative per mille (‰) difference between the sample and a recognised international standard (Canyon Diablo Troilite) at the Iso-Analytical Laboratory (UK) on a continuous-flow isotope ratio mass spectrometer. From 3 to 20 mg of each sample (giving ~40 µg of S)

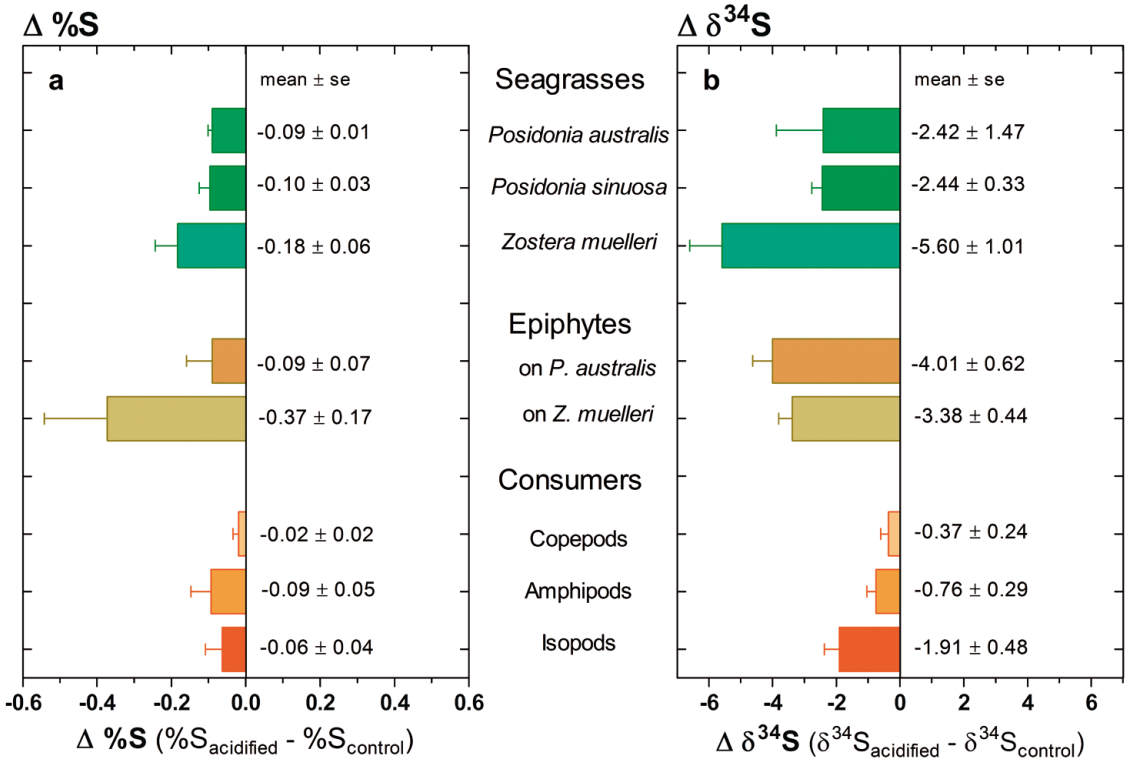

Fig. 2. Effects of acid treatment on (a) the elemental sulfur content and (b) the sulfur isotope ratio ($\delta^{34}S$) in biological sample material from seagrass meadows (epiphytes refers to algae growing on seagrass leaves). Negative values denote (a) lower concentrations of sulfur in the tissue following acidification, or (b) lighter isotope ratios resulting from depletion of ^{34}S following acid treatment

was weighed into tin capsules and vanadium pentoxide catalyst added. Analysis of multiple sub-samples (n = 3) demonstrated good precision (SE 0.14‰).

RESULTS

Acid treatment significantly altered S isotope ratios, causing a shift towards more depleted $\delta^{34}S$ values (paired *t*-test, $p < 0.001$; Fig. 2b). The mean ± SE shift across all groups was −2.61 ± 0.40‰, with individual samples becoming depleted by up to about 7‰. Changes in $\delta^{34}S$ attributable to sample treatment were greatest in the seagrass *Zostera muelleri*, followed by epiphytes and the 2 species of *Posidonia* (Fig. 2b). Although S isotope ratios in consumers were, on average, comparatively less affected by acid treatment, individual samples of isopods were depleted

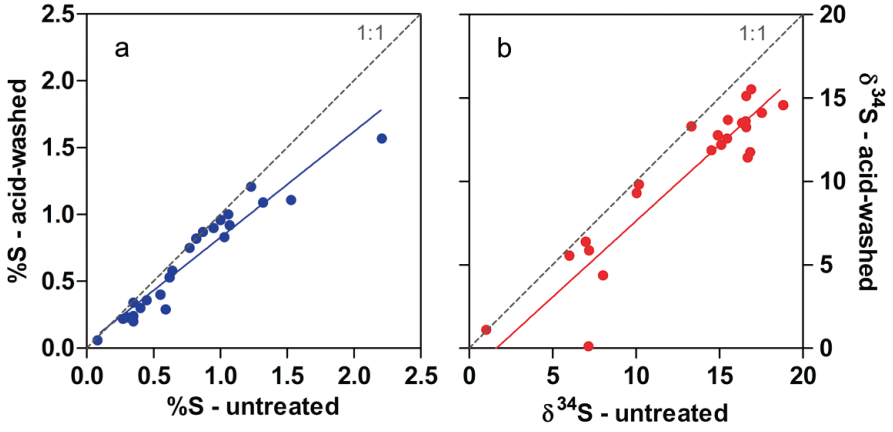

Fig. 3. Relationship between (a) sulfur content and (b) isotope ratios ($\delta^{34}S$) in acidified samples (*y*-axis) compared with paired aliquots that had not been exposed to acids (*x*-axis). Line of best fit is linear regression, significant in both panels ($p < 0.05$)

by up to nearly 3‰ in $\delta^{34}S$ following acidification. When examined over the full range of $\delta^{34}S$ values obtained, isotope shifts did not vary systematically in relation to the original $\delta^{34}S$ value of the untreated sample: relatively light samples were just as likely to become depleted due to acid treatment as more enriched samples (Fig. 3b).

The elemental S content of sample materials averaged 0.78 ± 0.10%. Epiphytic algae generally had the highest mean S content: it was 1.60 ± 0.33% in samples collected from the seagrass *Zostera muelleri* and 1.11 ± 0.17% in samples obtained from *Posidonia australis*. The lowest S content was recorded in the tissues of the 3 seagrass species (*Z. muelleri*: 0.51 ± 0.06%; *P. australis*: 0.42 ± 0.10%; *P. sinuosa*: 0.36 ± 0.05%). S concentrations in animal tissues were intermediate between epiphytes and seagrasses (isopods: 0.98 ± 0.06%; amphipods: 0.58 ± 0.28%; copepods: 0.71 ± 0.18%).

Acidification resulted in a significant decrease in tissue S content (paired *t*-test, $p < 0.001$; Fig. 2a). Concentrations of elemental S in acid-treated material were lower by 0.02 to 0.64% (mean decrease: −0.13 ± 0.03%; Fig. 2). These losses represent, on average, 17% of the total elemental S contained in the tissues before acid treatment; in individual samples, acidification removed up to half of the tissue S pool. The effects of acid-digestion on S concentrations were greatest in epiphytes of *Zostera muelleri*, followed by the tissues of the seagrasses *Z. muelleri* and *Posidonia sinuosa*. Loss of S during acidification was largely proportional to the original S content of the samples, albeit showing a slight tendency for some particularly S-rich samples to lose more S (Fig. 3a).

DISCUSSION

Inorganic compounds are not usually assimilated by consumers, and thus cannot affect the isotope ratios of consumers. Accurate modelling of nutrition pathways in food webs therefore relies on obtaining the isotope ratio of only the organic fraction of biological samples. Some components of aquatic food webs are either small, precluding simple excision of muscle tissue, or in the case of plants, are difficult to separate from associated sediment, or have attached biota that have carbonate skeletons. Such samples are normally acidified during laboratory processing to remove the inorganic C prior to combustion for mass spectrometry. A critical assumption of this acidification step is that it removes inorganic C, but does not cause substantial losses of C, N or S bound up in the organic fraction. By contrast, we observed losses of S in all tissue types following acidification. Similar decreases in elemental C attributable to acid treatment have been reported for phytoplankton (King et al. 1998), littoral invertebrates (Serrano et al. 2008, Vafeiadou et al. 2013) and marine sediments (Lohse et al. 2000), and have been used to indicate leaching or volatilisation of organic matter beyond the intended removal of inorganic C.

The general effect of acid treatment was a shift towards more negative $\delta^{34}S$ values in all tissue types examined. The magnitude of this method-related bias was variable, however, both within and among the categories of tissues tested. This variability in observed effect size (notwithstanding its overall strength and consistent direction) precludes the proposition of an 'acid correction term' that may be more widely applicable. Extrapolation of results beyond the taxa tested here should not be assumed and would require further testing. In addition, shifts in $\delta^{34}S$ caused by acid treatment were only weakly correlated with corresponding shifts in the elemental S content of the samples ($r = 0.37$, $p = 0.07$); they were also not significantly correlated with either the S content ($r = -0.13$, $p = 0.53$) or the S isotope ratio ($r = -0.22$, $p = 0.29$) of the untreated sample. Thus, neither the chemical composition nor the isotopic signature of untreated samples is an adequate or useful predictor of the magnitude of expected acid-related shifts in $\delta^{34}S$.

Acid effects on stable C isotope ratios have been reported for a range of biological materials (e.g. Jacob et al. 2005, Jaschinski et al. 2008, Mateo et al. 2008). However, no unequivocal explanation of the mechanisms that produce the observed shifts in isotope ratios has been reported in the literature. Processes that have been posited as mechanisms for isotopic change and fractionation of C and N caused by acid treatment—and which may equally well apply for S—include the following: (1) loss of acid-soluble organic matter during carbonate dissolution ('solubilisation') and leaching from tissues (Fernandes & Krull 2008, Serrano et al. 2008, Brodie et al. 2011); (2) volatilisation of organic compounds (Lohse et al. 2000, Brodie et al. 2011); (3) structural disintegrations of cells and tissues and break-up of complex compounds that are subsequently lost in sample rinses (Mateo et al. 2008); and (4) selective preservation or loss of organic matter and compounds with isotopically different signatures (Benner et al. 1987, Brodie et al. 2011). A fifth factor, loss of material during sample transfers, filtrations, rinses or decanting following acid treatment (Fernandes & Krull 2008, Brodie et al. 2011), is un-

likely to have been important in the current study since we did not rinse or wash samples with water.

Sulfur is a vital constituent of key structural and functional molecules such as amino acids, sulfolipids and cofactors such as vitamins and enzymes (Trust & Fry 1992, Brosnan & Brosnan 2006). The S content of plants typically ranges between 0.1 and 1.5% by dry weight (Trust & Fry 1992). In marine taxa, S is an essential element in dimethylsulfoniopropionate (DMSP), a secondary metabolite that is widespread in algae, vascular plants, invertebrates and vertebrates (Van Alstyne & Puglisi 2007). DMSP has a wide range of ecophysiological functions, being an antioxidant, a cryoprotectant, an osmolyte, a precursor to an activated defence system and a source of S for marine bacterioplankton (Van Alstyne & Puglisi 2007, Oduro et al. 2012). The S isotope ratio of DMSP in marine algae may be depleted in ^{34}S relative to other cellular components, since it is has different biochemical pathways to formation. We found no direct determinations of whether the S isotope ratio of DMSP differs from those of other cellular components, although it is known to differ from that in source seawater sulfate by 1 to 3‰ (Oduro et al. 2012). Selective removal of DSMP during acid treatments remains as an untested but possible mechanism for the isotopic changes that we observed (Figs. 1 & 2).

CONCLUSIONS

Acid treatment of biological material prior to isotope ratio mass spectrometry results in significant changes to the S content and isotopic composition of the samples. These changes represent a method bias that introduces avoidable error to food web analyses using SIs of S as chemical tracers. Thus, acid treatment should be avoided as a pre-analysis step in the measurement of $\delta^{34}S$, and determinations should made on untreated aliquots for materials where carbonate removal by acids is required for carbon isotope analysis.

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