

## Effects of acid treatment on carbon and nitrogen stable isotope ratios in ecological samples: a review and synthesis

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### Summary

1. Stable isotopes of carbon and nitrogen are mainstay tracers in diverse fields of ecology, particularly in studies of food webs.
2. Investigators are generally interested in tracing dietary C and N, and hence routinely remove non-dietary, inorganic C contained in calcified structures (e.g. shells, bones) by chemical dissolution of the carbonates. Acid treatment can, however, isotopically fractionate samples if part of the organic matter is lost or chemically modified, resulting in potentially altered  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values.
3. Here, we synthesize the effects of acid treatments on stable isotope analysis reported in the literature, showing that: (i) the method can change both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values; (ii) shifts in  $\delta^{13}\text{C}$  are generally, but not always, consistent with expectations of more depleted carbon ratios after the removal of the isotopically heavier inorganic carbonates; (iii) nitrogen ratios either decrease or increase in  $^{15}\text{N}$  content; and (iv) the majority (74–79% of comparisons) of reported changes to  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values attributable to acid treatment are  $<1\text{‰}$ , but larger acid effects can occur.
4. Acidification is needed if mechanical removal of calcified structures is unfeasible in carbonate-rich sample matrices containing low organic C and N, but should otherwise be very carefully considered before its use as a routine pre-treatment step of biological samples in isotope ratio mass spectrometry.

**Key-words:** inorganic carbon removal, stable isotope analysis, food webs, sample preparation, methods

### Introduction

In die Ecke, Besen, Besen! Seids gewesen, denn als Geister ruft euch nur zu seinem Zwecke erst hervor der alte Meister!	Back now, brooms, into the closet! Be thou as thou wert before! Until I, the real master call thee forth to serve once more!
Der Zauberlehrling (Johann Wolfgang von Goethe, 1797)	The Sorcerer's Apprentice

Only the technologically most recalcitrant of ecologists would wish for the broom of stable isotope (SI) techniques, a

broom that continues to forcefully sweep the discipline, to be confined to a closet. On the contrary, stable isotope applications in ecology – commonly used as biochemical tracers, tags or markers – encompass a remarkably broad ambit of themes. Recurrent objectives addressed by the use of stable isotopes in ecology are as follows:

1. To identify the sources, transport routes and processing of organic matter in detrital and sediment compartments (Schlacher & Wooldridge 1996; Middelburg *et al.* 2000; Oakes, Eyre & Middelburg 2012);
2. To delineate the key structural and functional traits of food webs (Fry 1988; Bunn, Davies & Winning 2003; Lee, Lee & Connolly 2012);
3. To recognize important dietary items and principal prey species of animal consumers (Riera *et al.* 1996; Vander Zanden & Vadeboncoeur 2002; Waltham & Connolly 2006; Fry *et al.* 2008);
4. To model the contributions of different sources to elements assimilated into consumer tissues (Phillips & Gregg 2001; Connolly, Hindell & Gorman 2005; Pitt *et al.* 2008; Parnell *et al.* 2010; Fry 2013);

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5. To determine the trophic position of consumers and the length of food chains (Cabana & Rasmussen 1994; Post 2002; Layman *et al.* 2012);

6. To trace the fluxes of organic matter and nutrients across ecosystem and habitat boundaries (LeBlanc 1989; Kato, Iwata & Wada 2004; Guest *et al.* 2006; Connolly, Schlacher & Gaston 2009; Schlacher & Connolly 2009; Schlacher *et al.* 2009);

7. To map nutrient and toxicant signals and measure their propagation through food chains exposed to contaminants (Van Dover *et al.* 1992; McClelland, Valiela & Michener 1997; Schlacher *et al.* 2005; Schlacher, Mondon & Connolly 2007; Pitt, Connolly & Maxwell 2009; Watanabe, Kodama & Fukuda 2009; Carmichael *et al.* 2012; Connolly *et al.* 2013);

8. To reconstruct historical diets (O'Donnell *et al.* 2003; Hedges & Reynard 2007; Jones, Walsh-Haney & Quinn 2012);

9. To track the movements of animals (Hobson 1999; Rubenstein & Hobson 2004; Zbinden *et al.* 2011);

10. To reveal the history of past ecosystems changes (Vander Zanden *et al.* 2003; West *et al.* 2006);

11. To reconstruct paleoclimates and paleoenvironments (Ivany *et al.* 2008; Yan *et al.* 2009); and

12. To determine the geographic origin of wildlife and other biological material in forensic applications (Van Der Merwe *et al.* 1990; Vogel, Eglinton & Auret 1990; Bowen, Wassenaar & Hobson 2005).

Ratios of the stable isotopes of carbon (i.e.  $^{13}\text{C}/^{12}\text{C}$ ) are the principal chemical tracer in many food web studies, chiefly because a consumer's tissue closely reflects the isotopic signature of its diet (McCutchan *et al.* 2003). In this context, ecologists are primarily interested in the organic fraction of carbon (OC) and generally wish to exclude the non-dietary, inorganic C (IC) from samples (Pinnegar & Polunin 1999). Such inorganic carbon consists mainly of carbonates that are precipitated into shells, teeth, bones and other skeletal structures of organisms. This IC – bound in calcified structures – may reflect the isotope signal of IC in the surrounding water or environment rather than carbon assimilated from the diet and represented as OC in the tissues (Yokoyama *et al.* 2005). Thus, analysis of the isotopic composition of organic carbon ( $\delta^{13}\text{C}$ ) in food web studies generally requires the removal of carbonates to obtain a trophic rather than an environmental signal.

Inorganic carbon is routinely removed by many investigators from biological samples prior to mass spectrometry because IC is isotopically heavier than most carbon of biological origin. Thus, even small amounts of IC can bias the  $\delta^{13}\text{C}$  values in samples analysed for stable isotopes of OC: for a hypothetical sample that contains 1% IC ( $\delta^{13}\text{C} \approx -3\text{‰}$ ) and 12% OC ( $\delta^{13}\text{C} \approx -25\text{‰}$ ) by weight,  $\delta^{13}\text{C}$  would be enriched by 1.8‰, the bias becoming greater with increasing amounts of IC in the sample (Brodie *et al.* 2011b).

The most popular technique for removing IC from samples is to expel IC as  $\text{CO}_2$  by acidification, usually with hydrochloric, sulphuric or phosphoric acid (Komada, Anderson & Dorfmeier 2008; Carmichael & Kovacs 2010; Kovacs *et al.* 2010; Versteegh, Gillikin & Dehairs 2011). An assumption of the technique is that acids dissolve inorganic carbonate associated with organic matrices containing carbon, but do

not otherwise chemically transform or result in loss of organic matter that fractionates stable isotope ratios. Although the exact mechanism(s) for potential acid-induced changes in elemental concentrations, isotope ratios, or both, remain undetermined in most cases for biological samples, many authors have suggested possible confounding effects caused by sample handling and chemical alterations by the acids itself (e.g. Froelich 1980; King *et al.* 1998). Furthermore, it is common to complement the analysis of carbon isotopes ( $\delta^{13}\text{C}$ ) with simultaneous measurements of nitrogen isotopes ( $\delta^{15}\text{N}$ ), chiefly to estimate trophic position (Post 2002). When samples are acid-treated to remove inorganic carbon, this pre-analysis step can have unintended consequences, resulting in altered nitrogen isotope ratios in some instances (Bunn, Loneragan & Kempster 1995; Bosley & Wainright 1999; Kennedy, Kennedy & Papadimitriou 2005).

Changes in stable isotope ratios that have been attributed to acid treatment of sample material are variable, and there is no consensus about effect sizes (e.g. Jacob *et al.* 2005; Jaschinski, Hansen & Sommer 2008; Mateo *et al.* 2008). Consequently, in this study we examine variability of 'acid effects' that have been reported in the literature, and we summarize effect sizes for different types of sample material commonly encountered by ecologists in food web studies of aquatic systems. Finally, we synthesize published recommendations about sample preparation routines that may involve acid treatment of sample material prior to isotope ratio mass spectrometry.

## Materials and methods

We extracted data on the effects of pre-analysis acid treatment from the primary, peer-reviewed literature, including papers that made explicit comparisons or tests of acid treatment on values of stable isotopes ratios of carbon, nitrogen, or both. Twenty-one studies were found that contained explicit tests on whether sample treatment with acids changes stable isotope ratios of carbon or nitrogen in aquatic samples (Table 1). From these 21 papers, a total of 297 numerical values were extracted that pertain directly to pairwise contrasts for carbon ( $n = 141$ ) and nitrogen ( $n = 156$ ). There were three main types of data that could be extracted, depending on how data were reported: (i) whenever available, we calculated contrasts from reported  $\delta$  values for matched aliquots of untreated and acid-treated samples, (ii) when individual sample values were not reported, contrasts were extracted or calculated from mean values of untreated and acid-treated samples, and (iii) in a few cases, contrasts were directly reported without information on either the sample values or means.

The types of sample material commonly included in stable isotope studies of aquatic systems for which data on acid treatment could be extracted from the literature comprised the following: sediment ( $n = 27$ ); suspended particulate organic matter, SPOM ( $n = 12$ ); cyanobacteria and biofilms ( $n = 12$ ); algae ( $n = 31$ ); seagrass ( $n = 2$ ); plankton ( $n = 16$ ); polychaetes ( $n = 8$ ); insects ( $n = 16$ ); peracarid crustaceans ( $n = 22$ ); decapod crustaceans ( $n = 56$ ); bivalves ( $n = 10$ ); gastropods ( $n = 32$ ); other molluscs ( $n = 8$ ); other invertebrates ( $n = 3$ ); and fish ( $n = 42$ ).

Samples were acidified most often with HCL, either by soaking, rinsing or washing them in acid (11 studies), applying the acid directly in

**Table 1.** Summary of data coverage used to review the effects of pre-analysis acid treatments on stable isotope ratios of carbon and nitrogen in ecological and environmental studies (If studies used more than one technique for different materials then these are listed separately; hence, row totals can be greater than the number of studies reviewed)

Total number of studies:	21*			
Total no records	297	Carbon: 141	Nitrogen: 156	
Preservation techniques:	Frozen 7	Dried/dry/freeze-dried 9	NA/other 6	Fresh 3
Acid type:	H <sub>2</sub> SO <sub>4</sub> 1	H <sub>3</sub> PO <sub>4</sub> 1	HCL 25	HCL/PtCl <sub>2</sub> 1
Application method:	Bathed/soaked/rinsed/washed 11	In capsule/direct 7	Fume exposure 2	NA/variable 4
Post-acidification rinse	Yes 9	No 12	NA 1	
Inferential statistics:	ANOVA 9	<i>t</i> tests 5	NA 5	Nonparametric 2

\*Sources: (Bunn, Loneragan & Kempster 1995), (Bosley & Wainright 1999), (Pinnegar & Polunin 1999), (Lorrain *et al.* 2003), (Perga & Gerdeau 2003), (Jacob *et al.* 2005), (Kennedy, Kennedy & Papadimitriou 2005), (Yokoyama *et al.* 2005), (Carabel *et al.* 2006), (Yokoyama & Ishihi 2006), (Ng, Wai & Williams 2007), (Fernandes & Krull 2008), (Jaschinski, Hansen & Sommer 2008), (Serrano *et al.* 2008), (Kolasinski, Rogers & Frouin 2008), (Mintenbeck *et al.* 2008), (Syväranta *et al.* 2008), (Dang *et al.* 2009), (Mazumder *et al.* 2010), (Ventura & Jeppesen 2010), (Brodie *et al.* 2011a).

the capsules on dried, milled material ( $n = 7$ ), or by exposure to acid fumes ( $n = 2$ ; Table 1). Twelve studies used a water rinse after acidification, while nine did not rinse samples following acid treatment (Table 1). Sample material was most often preserved by drying ( $n = 9$ ) or freezing ( $n = 7$ ).

Most (16 of 21) studies used inferential statistics to test for acid effects on isotope ratios. The detail of reporting was, however, not always sufficient to determine the significance of comparisons. This level of statistical under-reporting affected 28% ( $n = 40$ ) of carbon contrasts and 33% ( $n = 52$ ) of nitrogen contrasts.

Our chief objective was to examine the effects of acid treatment on SI values in samples commonly measured in aquatic ecology. Other aspects of sample treatment and preparation methods can also influence SI values, but are outside the scope of this study. These include preservation methods and chemicals (Fanelli *et al.* 2010; Xu *et al.* 2011; Lau, Leung & Dudgeon 2012; Rennie, Ozersky & Evans 2012), freeze/thaw periods and drying methods (Lorrain *et al.* 2003; De Lecea, Smit & Fennessy 2011b), lipid extraction (Logan *et al.* 2008; Mintenbeck *et al.* 2008), the type of capsule material (Bosley & Wainright 1999; Brodie *et al.* 2011a) and the use of dyes (De Lecea *et al.* 2011a). We also acknowledge the limitations of the meta-analytical approach: individual studies included in our synthesis differ with respect to the types of acids used, concentrations, incubation times, the number and duration of washes, and the type of mechanical sample handling; whilst these variants may influence effect sizes of reported changes in isotope ratios broadly attributable to acid treatment, the chief intention of our synthesis is to derive generalities broadly applicable to a range of biological samples.

## Results

Acidification altered isotope ratios in the majority of samples (Table 2, Fig. 1). Acid treatment lowered carbon isotope ratios by a mean of 0.68 ‰ (SE 0.12), and nitrogen isotope ratios by a mean of 0.16 ‰ (SE 0.06). Acid effects on  $\delta^{13}\text{C}$  encompassed a broad ambit, ranging from shifts towards lighter values up to  $-8.09$  ‰, to enrichments up to  $1.90$  ‰.

Nitrogen isotope ratios became up to  $-3.22$  ‰ depleted and up to  $2.90$  ‰ enriched following acid treatment. Variability in acid-induced isotope changes was greater for  $\delta^{13}\text{C}$  (interquartile range =  $0.97$  ‰) than for  $\delta^{15}\text{N}$  ( $0.63$  ‰; Fig. 1).

More than two-thirds (68 %,  $n = 96$ ) of carbon samples were more negative after acid treatment; of these just under half (48 %,  $n = 46$ ) were significant contrasts (Table 2). A greater number of comparisons of nitrogen isotope ratios also shifted towards more depleted ratios (55 %,  $n = 86$ ), but there was also a sizeable proportion (42 %,  $n = 66$ ) of more enriched values after acid treatment (Table 2); only 11 contrasts for  $\delta^{15}\text{N}$  were reported as significant (Table 2).

The magnitude of acid effects differed between carbon and nitrogen, being generally greater for the former (Table 2). For carbon, half of all comparisons showed changes in  $\delta^{13}\text{C}$  smaller than  $0.5$  ‰ and three-quarters of acid-related effects were smaller than  $1$  ‰ (Table 2). We found only six comparisons where isotope ratios changed by more than  $4$  ‰ following acid treatment. All of these samples were more depleted after acid treatment and contained calcified structures (i.e. exoskeleton of shrimp, Yokoyama *et al.* 2005; red macroalgae, Ng, Wai & Williams 2007) or had high carbonate content (i.e. marine sediment and SPOM, Carabel *et al.* 2006).

Absolute shifts in  $\delta^{15}\text{N}$  due to acid treatment were smaller than those recorded for carbon: in two-thirds of comparisons,  $\delta^{15}\text{N}$  differed by  $<0.5$  ‰ and was changed by  $<2$  ‰ in 95 % of records (Table 2). Negative shifts in N-isotope ratios following acid treatment greater than  $2$  ‰ were recorded in four samples of macroalgae (Ng, Wai & Williams 2007), cyanobacteria and biofilm (Ng, Wai & Williams 2007), and a portunid crab (Carabel *et al.* 2006). Bunn, Loneragan & Kempster (1995) found a  $^{15}\text{N}$ -enrichment effect of  $2.9$  ‰ in the tail muscle of shrimp after acidification.

The size of the acid effect on  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values varied between sample types (Fig. 2). Carbon isotope ratios became

**Table 2.** Summary of changes to  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of sample material treated with acids

A – Direction of change	Carbon			Nitrogen		
	Negative	No change	Positive	Negative	No change	Positive
Number of contrasts	96 (68%)	5 (4%)	40 (28%)	86 (55%)	4 (3%)	66 (42%)
Significance of contrasts						
$P < 0.05$ ('yes')	46 (48%)	–	0 (0%)	23 (27%)	–	11 (17%)
$P > 0.05$ ('no')	24 (25%)	–	26 (65%)	39 (45%)	–	27 (41%)
NA	26 (27%)	–	14 (35%)	24 (28%)	–	28 (42%)

B – Magnitude of change	Carbon			Nitrogen		
	Size of change in $\delta^{13}\text{C}$ (‰)	<i>n</i>	%	Size of change in $\delta^{15}\text{N}$ (‰)	<i>n</i>	%
	<0.5	75	53	<0.5	103	66
	<1.0	104	74	<1.0	124	79
	<2.0	127	90	<2.0	148	95
	<3.0	130	92	<3.0	155	99
	<4.0	135	96	<4.0	156	100

NA, not available, meaning that too little information was available to test differences statistically.

most strongly depleted in sediments, followed by suspended particulate organic matter, cyanobacteria, crustaceans and algae (Fig. 2). The  $\delta^{13}\text{C}$  values of molluscs, insects, polychaetes and fish tissue were less strongly altered by acid treatment (Fig. 2). Samples of sediment and SPOM also showed considerable variation for acid effects on their  $\delta^{15}\text{N}$  values (Fig. 2). In terms of mean shifts,  $\delta^{15}\text{N}$  values of cyanobacteria, algae and some molluscs were most heavily affected by acid treatment, whereas nitrogen isotope ratios of insects, crustaceans and fish tissue were not strongly altered (Fig. 2).

## Discussion

### CHANGES IN $\delta^{13}\text{C}$ AND $\delta^{15}\text{N}$ FOLLOWING ACID TREATMENT

A number of processes have been posited to cause, or contribute to, observed changes in stable isotope ratios, due to either effects caused by sample handling, chemical alteration by the acid, or instrument measurement errors. Specifically these include:

1. Loss of acid-soluble organic carbon during carbonate dissolution – 'solubilization' (Froelich 1980; Fernandes & Krull 2008; Brodie *et al.* 2011b);
2. Unconfined effervescence, which may catapult droplets out of the sample cup or capsule (Lohse *et al.* 2000);
3. Break-up of complex compounds and subsequent loss in rinses (Mateo *et al.* 2008);
4. Structural disintegrations of cells and tissues (Mintenbeck *et al.* 2008);
5. Leaching from tissue (Goering, Alexander & Haubenstein 1990; Serrano *et al.* 2008);
6. Volatilization of organic compounds (King *et al.* 1998; Lohse *et al.* 2000; Brodie *et al.* 2011b);
7. Loss of material (e.g. very fine particles, organic colloids) during post-treatment sample transfers, filtration, rinses or

decanting (King *et al.* 1998; Fernandes & Krull 2008; Brodie *et al.* 2011b);

**8.** Fractionation of organic matter during acid evaporation (King *et al.* 1998; Lohse *et al.* 2000; Kennedy, Kennedy & Papadimitriou 2005; Fernandes & Krull 2008);

**9.** Preservation of organic matter with a light  $\delta^{13}\text{C}$  signature, possibly due to preferential retention of acid-insoluble lipids and lignin known to be depleted in  $^{13}\text{C}$  (Benner *et al.* 1987; Schubert & Nielsen 2000; Brodie *et al.* 2011b);

**10.** Poor analytical precision due to low C or N recovery in sample material and inadequate calibration or baseline correction (Fry *et al.* 1992; Carmichael *et al.* 2008).

There are several reports of sizeable reductions in the elemental C and N content by weight following demineralization using acids. For example, acid treatment has been shown to lower the elemental N content by 0.9–1.9% in littoral invertebrates (Serrano *et al.* 2008), 7–49% in marine sediments (Lohse *et al.* 2000) and 0.2–0.25% in phytoplankton (King *et al.* 1998). It appears plausible that the observed shifts in isotope ratios can be accounted for, at least partly, by the removal of organic C and N fractions following acid treatment, either through preferential loss of particular C and N forms or through a decrease in elemental recovery to levels below accurate analytical determination.

It is assumed that demineralization with acids removes carbon that forms part of inorganic, non-dietary carbonates deposited in skeletal material, shells and other calcified structures. However, the possibility that carbon not bound up in carbonates is also removed by acid washing exists. For example, Serrano *et al.* (2008) found significant changes in the elemental C and N content in beach arthropods that were not accompanied by corresponding changes in isotope ratios. They argue that this implies preferential removal of organic carbon compounds that are comparatively depleted in  $^{13}\text{C}$  to counterbalance the loss of the  $^{13}\text{C}$ -enriched carbonates. Amongst the many possible candidate carbon compounds that

**Table 3.** Summary of key recommendations about acid treatment of samples intended for stable carbon and nitrogen analysis in ecological studies given by individual authors, ordered by type of sample material/taxon

References	Sample Material	Recommendations	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
(Fernandes & Krull 2008)	Sediment	<i>“The fact that all acid treatments led to significant deviations in <math>\delta^{15}\text{N}</math> values indicates that it is crucial to obtain nitrogen isotopic signatures from untreated aliquots, analysed in the so-called ‘single mode’ (as opposed to ‘dual mode’ isotopic analysis).”</i>	YES (sediments)	NO
(Brodie <i>et al.</i> 2011a,b)	Sediment	<i>‘Samples should be processed and analysed for C and N on separate aliquots, in the knowledge of the potential for acid-induced bias.’</i>	NA	NO
(Lorrain <i>et al.</i> 2003)	Suspended Particulate Organic Matter (SPOM)	<i>‘Decarbonation is necessary to remove all inorganic carbon from samples before measuring POC.’</i>	YES	NA
(Carabel <i>et al.</i> 2006)	Sediment, SPOM, Macroalgae, Zooplankton (mixed), Decapod Crustaceans, Cephalopods, Fish	<i>‘Samples should not be acidified for nitrogen isotope analysis, since a decrease in <math>^{15}\text{N}</math> was observed for some groups after this treatment. Acidification for carbon would not be necessary in seaweeds, fish muscle and cephalopods. Acidification is needed for carbon stable isotope analysis in samples of sedimentary OM, SPOM, invertebrates with calcareous structures and plankton.’</i>	NO/YES for sediment, plankton, and invertebrates with calcareous structures	NO
(Ng, Wai & Williams 2007)	Macroalgae, Chitons, Gastropods	<i>‘It is therefore recommended to only acid-wash carbonate-rich algal samples, especially crustose forms.’</i>	NO/YES only for carbonate-rich algae	NA
(Jacob <i>et al.</i> 2005)	Invertebrates (92 species). Fish (11 species)	<i>‘Acidify samples prior to stable isotope analysis by careful application of as little HCl as possible. Apply 1 mol l<sup>-1</sup> HCl drop-by-drop until no further CO<sub>2</sub> development is visible.’</i>	YES	YES
(Jaschinski, Hansen & Sommer 2008)	Crustaceans, Gastropods	<i>‘Our results suggest that the used in situ acidification method can be applied to eliminate non-dietary carbon without significantly influencing the chemical composition of the studied samples. Nevertheless, we recommend that sample only be acidified if absolutely necessary.’</i>	NO/YES only if ‘absolutely necessary’	NO
(Serrano <i>et al.</i> 2008)	Insects, Spiders, Centipedes, Crustaceans	<i>‘In order to avoid isotopic variability due to acid washing samples, we recommend using non-acidified soft tissues whenever possible for isotopic analyses.’</i>	NO/YES only for invertebrates with carbonate proxy >0.09 using ‘soft’ acid treatment	NO
(Kolasinski, Rogers & Frouin 2008)	Polychaetes, Crustaceans, Molluscs, Anemones	<i>‘Samples with a carbonate proxy &gt;0.05 must be acidified, while acidification efficiency should be tested for taxa with carbonate proxies which lie between 0.03 and 0.05. Acid pre-treatment should be avoided for tropical taxa which have no carbonate content (carbonate proxy &lt;0.03).’</i>	NO/YES depending on carbonate content	Either

Table 3. (Continued).

References	Sample Material	Recommendations	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
(Yokoyama & Ishihi 2006)	Bivalves	<i>'In cases where there is a possibility of contamination of carbonates such as fragments of the shell, acid treatment for a short time (&lt;3 min) followed by brief rinsing of the total soft tissues prior to grinding will be allowed.'</i>	NO/YES if carbonate contamination suspected/possible	NA
(Bunn, Loneragan & Kempster 1995)	Decapod Crustaceans	<i>'Little justification for acid-rinsing samples of shrimp tail muscle.'</i>	NO	NO
(Yokoyama <i>et al.</i> 2005)	Decapod Crustaceans (ghost shrimp)	<i>'Acid treatment on muscle samples (for crustaceans) would be unnecessary, unless debris of exoskeletons or any other inorganic carbonates are included in the samples.'</i>	NO/YES only if inorganic carbonate present	NA
(Mazumder <i>et al.</i> 2010)	Decapod crustaceans	<i>'We recommend that acid washing of muscle samples be avoided unless absolutely necessary.'</i>	NO	NO
(Bosley & Wainright 1999)	Decapod Crustaceans, Fish	<i>'Our results suggest that acidification may be an unnecessary preparation step for at least some types of animal tissue samples, including some that have exoskeletons.'</i>	NO	NO
(Pinnegar & Polunin 1999)	Fish	<i>'Sensible approach would seem to be to try to select tissues with low amounts of hard structural components in order to reduce contamination of <math>^{13}\text{C}</math> analysis by inorganic carbonates, and not necessitating acid treatment.'</i>	NO/YES only if exclusion of carbonate-rich tissues is not feasible	NO

could be preferentially leached by acid attack, chitin ( $\delta^{13}\text{C} = -23.6\text{‰}$ ) may be important, provided that it undergoes depolymerization and deacetylation (Percot, Viton & Domard 2003). A similar mechanism (i.e. partial loss of cuticle matrix compounds) may explain some of the observed changes in  $\delta^{15}\text{N}$  (Serrano *et al.* 2008), but this has not been tested chemically or with compound-specific isotope analysis.

Organic carbon (OC) molecules, or functional C groups, that are more acid-labile (e.g. carbohydrates, amino acids) may be more enriched in  $^{13}\text{C}$  compared with lipids and non-hydrolysable material (Hwang & Druffel 2003). Such asymmetrical susceptibility to hydrolysis between OC compounds that differ in  $^{13}\text{C}$  could therefore contribute to isotopic changes in samples following acid treatment; these isotopic changes could occur in addition to any effects caused by the removal of IC. Because the objective of acid treatment is to remove IC without altering the OC, this 'isotopic fractionation' could introduce inaccuracies to the analyses (Komada, Anderson & Dorfmeier 2008). Since hydrolysable OC has a greater N content relative to the bulk material (Hwang *et al.* 2006), similar effects of acid treatments are possible for N isotopes.

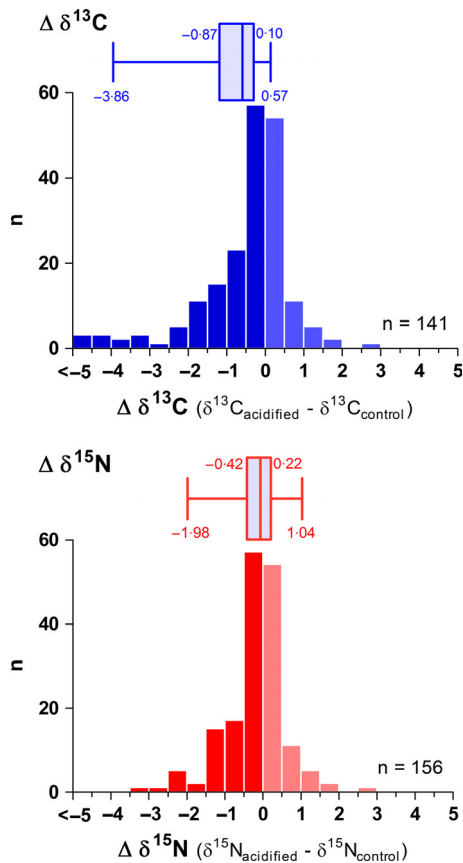
Organisms can contribute substantially to sedimentary carbon pools through their calcified structures ('biogenic  $\text{CaCO}_3$ '). Biogenic  $\text{CaCO}_3$  contains organic matter as glycoproteins, synthesized by the organism to aid mineral

precipitation and is incorporated into the mineral during calcification (Ingalls *et al.* 2004). Because up to two-thirds of compounds constituting these mineral-bound glycoproteins are acid-soluble (Ingalls *et al.* 2004), it is not implausible that some of this organic matter is lost during acid treatment of SI samples (Fernandes & Krull 2008).

#### CARBONATE-RICH SAMPLES: THE 'CHAMPAGNE TEST' & CARBONATE PROXY

The effect of non-dietary IC in  $\delta^{13}\text{C}$  analysis depends mainly on the carbonate content of the samples: material that contains non-trivial amounts of inorganic carbon usually requires decalcification prior to mass spectrometry (Jacob *et al.* 2005; Carmichael *et al.* 2008). Carbonate-rich samples that are commonly encountered in ecological studies encompass sediments, algae with calcified structures (and encrusting epibionts), bryozoans, shelled molluscs, crustaceans, echinoderms, and whole fish or fish-larvae analysed *in toto*.

Whether a sample has high IC content can in many cases be predicted from basic morphological and physiological properties of the material (e.g. molluscs with shells, carbonate-rich marine sediments). A complementary and practicable alternative is to determine the presence of carbonates in samples by applying drops of acid to dried material and examining whether bubbles form (i.e.  $\text{CO}_2$  produced by the chemical



**Fig. 1.** Shifts in  $\delta^{13}\text{C}$  (top panel) and  $\delta^{15}\text{N}$  (bottom panel) of sample material following acid treatment to remove inorganic carbon prior to mass spectrometry. Each value represents a contrast between acid-treated and untreated samples reported in the literature. Whiskers in the box & whisker plots are the 5% and 95% percentiles, the boxes encompass the quartile range, and the middle line is the median.

dissociation of the carbonates). This ‘Champagne Test’ is a sensible complement if there are uncertainties about the carbonate content of the samples.

It seems sensible to exclude non-dietary carbonates from the analysis by physically removing calcified structures before combustions rather than introducing a chemical step in sample preparation which may have unintended effects on stable carbon isotope ratios. Acidification to remove non-dietary carbon is, however, required for species or individuals too small to allow for the effective removal of calcified structures (e.g. exoskeletons, shells, internal skeletal structures). Such small animals are usually crushed whole and acidification may be required for those known to have high inorganic carbonate content. Smaller calcareous structures (e.g. spicules, radulae) can also affect  $\delta^{13}\text{C}$  values. If these cannot be removed manually, acid treatment may be required in cases where these structures are likely to contribute significantly to the total carbon pool of the material.

Jacob *et al.* (2005) introduced a surrogate for the direct chemical measurements of the inorganic carbonate content ( $\text{CaCO}_3$ ) in animal tissues, termed the ‘carbonate proxy’. It is calculated as the relative change in the C : N ratio following acidification (Carbonate proxy =  $[(\text{C} : \text{N})_{\text{crude}} /$

$(\text{C} : \text{N})_{\text{acidified}}] - 1$ ) and appears to be a practicable and useful tool to estimate the likelihood of samples having large mineralized C components. Shifts in  $\delta^{13}\text{C}$  that can be accounted for by acid treatment show a relationship with the carbonate proxy as predicted from isotopic carbonate mass balance (i.e. loss of relatively  $^{13}\text{C}$ -enriched  $\text{CaCO}_3$  resulting in more depleted samples after acid treatment in samples of high carbonate content). Jacob *et al.* (2005) report that acid treatment had isotopic effects similar to the range of isotopic separations commonly reported between adjacent trophic levels (0.5–1.0‰) at carbonate proxy values as low as 0.08. Serrano *et al.* (2008) use a very similar carbonate proxy value of 0.09 to identify samples, which are more likely to require demineralization. Finally, Kolasinski, Rogers & Frouin (2008) found significant ( $P < 0.05$ ) acid-induced changes in  $\delta^{13}\text{C}$  values (mean shift of  $-0.59$ ‰) in polychaetes with a relatively low carbonate proxy value of 0.047; they recommend to acidify all samples with a carbonate proxy  $>0.05$ , test the effects of acidification for taxa with carbonate proxies between 0.03 and 0.05, and avoid acid pre-treatment for samples that have negligible carbonate content (defined as a carbonate proxy value  $<0.03$ ).

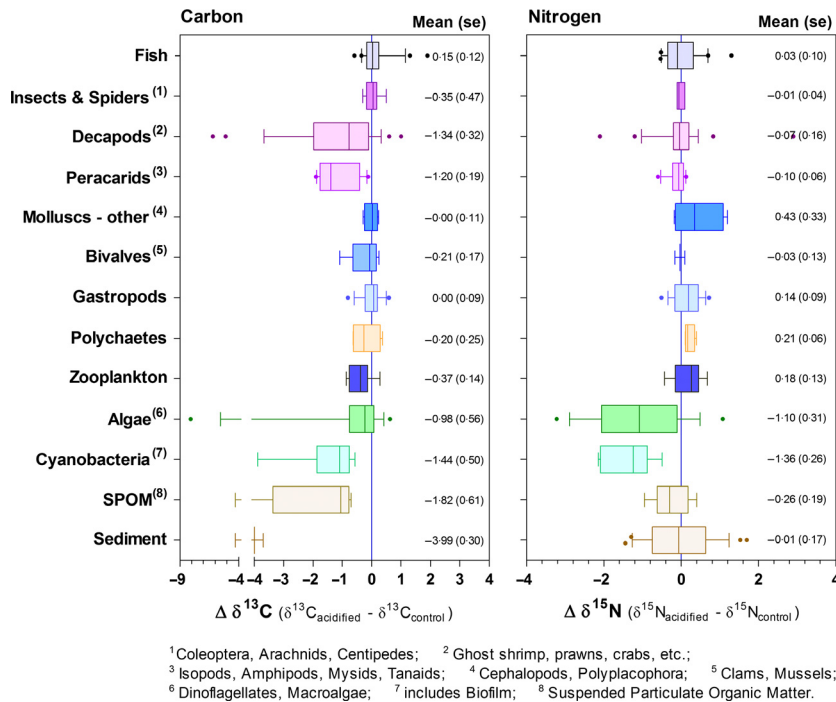
#### EFFECTS ON NITROGEN ISOTOPE RATIOS

It is common practice to measure both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  simultaneously in ‘dual mode’ analysis. Whilst chemical removal of inorganic carbon by acids can be warranted for samples with appreciable carbonate content, this treatment can lead to unintended changes in  $\delta^{15}\text{N}$  values measured on acid-treated material (Fig. 1).

Demineralization may, however, be necessary before  $\delta^{15}\text{N}$  measurements for samples that contain low amounts of N embedded in a carbonate matrix (e.g. mollusc shells; Carmichael *et al.* 2008; Versteegh, Gillikin & Dehairs 2011). In cases where acid treatment is the only practicable way to separate, or concentrate, the organic matter of interest from the inorganic carbonate matrix (e.g. bivalve shells) assessments of ‘acid effects’ lack true controls, but this can be overcome using proxy controls of matched soft tissues (Carmichael *et al.* 2008).

Versteegh, Gillikin & Dehairs (2011) showed for blue mussel shells that accurate  $\delta^{15}\text{N}$  values can be obtained for samples with as little as 20 mg N (or 1.6% N) when direct combustion is used. Although prior acidification may not be routinely required to measure nitrogen isotope ratios in all calcified material (Versteegh, Gillikin & Dehairs 2011), decisions regarding demineralization are best made on a case-by-case basis according to the N content of the samples and instrument capabilities.

Whether an acid effect becomes ‘significant’ ultimately depends on the question that a study addresses, the precision and power required to distinguish between treatments, the variability of the samples, and the isotope contrast in the source materials. For example, distinguishing the relative contribution of seagrass and upland C3 plants to the diet of an estuarine producer using  $\delta^{13}\text{C}$  could be relatively immune to acidification effects since source signatures are so widely



**Fig. 2.** Variation in changes to carbon (left panel) and nitrogen (right panel) isotope ratios following acid treatment between types of sample materials/taxa. Whiskers in the box & whisker plots are the 5% and 95% percentiles, the boxes encompass the quartiles, and the median is represented by a line inside the boxes.

separated (assuming a relatively homogeneous sample of consumers). By contrast, acid effects may become important when distinguishing between benthic microalgae and phytoplankton if these have similar  $\delta^{13}\text{C}$  signals; even in the latter case, acid effects are only of concern if the size and/or direction of the bias differs between sample types. The scenarios in which acid effects may or may not be an issue of concern are diverse and should be carefully considered by investigators when making methodological decisions.

#### SYNTHESIS AND RECOMMENDATIONS

1. Stable isotope ratios may be altered by chemical transformation or loss of organic matter that leads to fractionation (posited but unknown) or by loss of organic matter that results in poor analytical precision and accuracy.
2. Methods for acidification of samples differ between studies (Table 3), introducing uncertainty with regard to the comparability of carbon and nitrogen stable isotope ratios. Acidification should therefore always be very carefully considered before its use as a pre-treatment step in SI analysis.
3. Our review of acid treatment effects on carbon and nitrogen isotope ratios demonstrates that: (i) the method can alter the values of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , (ii) these changes are generally, but not always, consistent with expectations of more depleted carbon ratios after removal of isotopically heavy inorganic carbonates, (iii) bias is introduced to nitrogen ratios which either decrease or increase in  $^{15}\text{N}$  content, and (iv) the majority (74–79% of comparisons) of reported changes to  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values attributable to acid treatment are  $< 1\text{‰}$ , but larger acid effects can occur.
4. Instead of using acidification routinely, the chemical composition of sample material should be determined before performing inorganic carbonate removal with acids.

5. Carbonates contained in skeletal or other decalcified structures are best removed mechanically rather than by chemical dissolution. In cases where this is not possible, or where contamination with carbonate-rich sediments or other particles is suspected, acid treatment can be used to remove non-dietary inorganic carbon from biological material.

6. If acid treatment is required for carbon isotope samples, samples should be run in single-mode analysis where nitrogen isotope signatures are obtained from untreated aliquots of the material.

7. Investigators must ensure that acidification produces adequate recovery of organic matter to allow for reliable analytical precision, and report details on analytical methods and the quantity of material analysed.

8. In situations, where investigators are uncertain about the inorganic carbon content of their samples, are unable to mechanically remove calcareous material effectively, or suspect contamination with carbonates, the most sensible approach is to determine whether inorganic carbonate contamination exists by running acidified and untreated samples as a pilot batch before the full analysis. Using the ‘champagne test’ or the carbonate proxy may also be helpful in these situations.

9. Notwithstanding the broad applicability of the above suggestions, there may not be a universal protocol that applies in all situations – the prudent course of action is to analytically determine the effects of sample treatment on isotope ratios in all cases where method bias is suspected.

#### Data accessibility

All data pertaining to this publication are stored in accordance with USC’s ‘Research Data and Management Procedure’ (<http://www.usc.edu.au/university/governance-and-executive/policies-and-procedures/>)



research-data-and-materials-procedures). They are available directly from the first author (TA Schlacher, tschlach@usc.edu.au) or the USC Research Data Bank.

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