Rapid Communications in Mass Spectrometry

Received: 13 October 2010 Revised: 10 December 2010 Accepted: 13 December 2010 Published online in Wiley Online Library: 2011

Rapid Commun. Mass Spectrom. **2011**, *25*, 675–680
(wileyonlinelibrary.com) DOI: 10.1002/rcm.4905

Dear Editor,

Analysis of $\delta^{15}N$ values in mollusk shell organic matrix by elemental analysis/isotope ratio mass spectrometry without acidification: an evaluation and effects of long-term preservation

Nitrogen stable isotope ratios ($\delta^{15}N$) have successfully been applied in the study of trophic linkages,[1–3] as well as of human impacts in aquatic ecosystems. Anthropogenic wastewater input typically elevates $\delta^{15}N$ values in dissolved inorganic nitrogen and this ^{15}N enrichment subsequently propagates throughout the food chain.[4–7] Bivalve mollusks are of interest for studies of this human influence since they are primary consumers and are known to trace environmental $\delta^{15}N$ variability.[8,9] The $\delta^{15}N$ values of their soft tissues have, for example, been found to correlate with the fraction of residential development in watersheds around lakes and salt marshes.[10,11] To determine the 'undisturbed' $\delta^{15}N$ values in an ecosystem, before anthropogenic nitrogen input, $\delta^{15}N$ records need to be extended into the past. Bivalve shells can be useful for this, since they are often abundant in archaeological deposits as well as historic museum collections. A predictable relationship has been demonstrated between the $\delta^{15}N$ values of shell organic matter and soft tissues[12] and $\delta^{15}N$ values of this organic matrix indeed trace anthropogenic influences.[13–15]

Various sample preparation techniques have been used to analyze $\delta^{15}N$ values of skeletal organic matter, such as acidification[12–16] or simple combustion of whole skeletal material (without prior acidification).[17–19] These methods are also used in analysis of organic matter (e.g., soft tissues, particulate organic matter). Animal soft tissue samples contain varying amounts of $CaCO_3$, which will introduce a bias in $\delta^{13}C$ measurements. Therefore, samples are generally treated with an HCl solution before analysis.[13,14,20,21] However, the acidification process in itself may influence $\delta^{15}N$ values,[22–25] although some authors found no effect of acidification on $\delta^{15}N$ values.[26] Nevertheless, authors typically avoid acidification of samples for $\delta^{15}N$ analysis and will run one set of non-acidified samples for $\delta^{15}N$ and one for $\delta^{13}C$ (e.g., Bouillon *et al.*[21]). If there are no effects of $CaCO_3$ on $\delta^{15}N$ analysis, then avoiding acidification would be the method of choice for $\delta^{15}N$ analysis of shell organic matter. Moreover, direct combustion of shell material is easier and less time-consuming than acidification.

In museum collections bivalve shells are traditionally dry-stored, whereas soft tissues are preserved in 70% ethanol, sometimes after fixation with 10% formalin. However, often the whole animal is preserved in ethanol and shells are not stored separately. For the application of these preserved specimens in the investigation of past $\delta^{15}N$ values it is essential to know if liquid preservation methods have an effect on the $\delta^{15}N$ values of bivalve shells and if this effect is predictable. The effects of liquid preservation on the $\delta^{15}N$ values of biological tissues have been examined in a variety of animals. Syväranta *et al.* [27] found that neither formalin nor ethanol had a significant effect on $\delta^{15}N$ values of preserved zooplankton and macroinvertebrates. However, in fish muscle, enrichments of 0.5 to 1.4‰ have been found after fixation in formalin and subsequent preservation in ethanol[26,28,29] (Table 1). Results on mollusks differ among studies, but generally preservation effects on tissue $\delta^{15}N$ values are small in short-term studies. Sarakinos *et al.*[30] found that ethanol preservation lowered $\delta^{15}N$ values of the soft tissues of the freshwater bivalve *Corbicula fluminea* by −0.39‰ after 6 months. Similarly, in the freshwater mussel *Amblema plicata*, ethanol preservation for 1 year caused a change of −0.23‰ in tissue $\delta^{15}N$ values[31] (Table 1). In contrast, some other workers found higher $\delta^{15}N$ values for liquid-preserved mollusk tissue samples in comparison to frozen or dried samples. Ethanol preservation for 12 weeks resulted in a non-significant enrichment in octopus and Littorinid tissues.[32] In *Mytilus galloprovincialis* and *Patella vulgata*, tissue $\delta^{15}N$ values increased up to 1.1‰ and 1.0‰, respectively, after treatment with formalin for 2 days and ethanol for 6–24 months[33] (Table 1). In summary, wet preserved specimens typically exhibit a small enrichment in ^{15}N, but this effect is variable between studies (Table 1).

We report herein the evaluation of the method of simple combustion without acidification by testing the influence of $CaCO_3$ content on $\delta^{15}N$ values of different mixtures of acetanilide [$C_6H_5NH(COCH_3)$] and synthetic pure $CaCO_3$. We also investigate the fractionation between tissue and shell organic matrix in the bivalve *Mytilus edulis*. Finally, we examine the effects of long-term (73 years) ethanol preservation on $\delta^{15}N$ values of bivalve shell organic matrix.

For the comparison of $\delta^{15}N$ values of mantle tissue and shell organic matrix, three specimens of the blue mussel *Mytilus edulis* were collected in 2002 in Knokke, Belgium (salinity ~30; see Gillikin *et al.*[34] for site description). For the investigation of the long-term effect of ethanol preservation, six shells from the Royal Belgian Institute of Natural Sciences collected at Dudzele (Belgium, from a canal linked to the North Sea) on 27 March 1936 were selected. Three individuals were stored dry and three individuals were preserved in ethanol along with whole soft tissues. In addition, dry-stored shells from three individuals collected at a nearby site at Lissewege on 22 November 1938 were obtained from the same museum and one shell, collected on 3 June 1935 at Knokke, was obtained from the Dutch National Museum of Natural History, Naturalis.

All shell samples were rinsed with deionized water (>18 MΩ/cm) and left to dry. The periostracum was completely removed with a Dremel abrasive buff. Calcite samples were taken from the outside of the shell with a hand drill; the inner aragonite layer was avoided. Between 10 and 20 mg of calcite powder was collected, covering an area of at least 1 year of the most recent growth. The mantles from the ethanol-preserved specimens were dissected, rinsed with Milli-Q grade water and dried overnight at 60°C and pooled. An aliquot of the ethanol these specimens were preserved in

Table 1. Effects of formalin-ethanol and ethanol-only preservation on different animal tissues

Reference	Taxon/tissue	Preservation method	Effect on $\delta^{15}N$ values (‰)
Syväranta *et al.*[27]	Zooplankton, macroinvertebrates whole	Ethanol (12 months)	None
Arrington *et al.*[28]	Fish muscle	Formalin (2 weeks) + ethanol (6 weeks)	+0.62 (±0.04)
Bosley & Wainright[26]	*Pleuronectes Americanus* muscle	Formalin (2 months) + ethanol (2 months)	+1.41 (±0.22)
Edwards *et al.*[29]	Fish muscle	Formalin + ethanol (12–15 years)	+0.5
Sarakinos *et al.*[30]	*Corbicula fluminea all soft*	Ethanol (6 months)	−0.39
Delong & Thorp[31]	*Amblema plicata muscle*	Ethanol (12 months)	−0.23 (±0.03)
Kaehler & Pakhomov[32]	Octopus (arm), Littorinid (foot)	Ethanol (12 weeks)	Non-significant increase
Carabel *et al.*[33]	*Mytilus galloprovincialis*	Formalin (2 days) + ethanol (6–24 months)	+1.1
	Patella vulgata muscle		+1.0
This study	*Mytilus edulis* shell	Ethanol (73 years)	−5.2 to −5.9

was dried and the residue was analyzed. For testing the influence of $CaCO_3$ content on $\delta^{15}N$ measurements, different mixtures of acetanilide [$C_6H_5NH(COCH_3)$] with inorganic pure $CaCO_3$ (Merck pro analysis) were made, containing between 0 and 10.4 weight % N (or 100 to 0 weight % $CaCO_3$).

Powder calcite samples were loaded into 4 × 6 mm tin cups and weighed. $\delta^{15}N$ values were measured using an elemental analyzer (EA, Flash 1112 EA; ThermoFinnigan) coupled via a CONFLO III to a ThermoFinnigan Delta V^+ isotope ratio mass spectrometer (IRMS). An inline soda lime CO_2 trap was used to scrub CO_2 from the gas stream entering the gas chromatography column of the EA. IAEA-N1 was used as a standard, with an accepted value of 0.4 ± 0.2‰.[35] Long-term standard reproducibility is better than 0.1‰ for samples larger than 30 μg N (this typically increases to 0.2 or 0.3‰ with smaller samples between 10 and 30 μg N). $\delta^{15}N$ values are expressed in ‰ vs. atmospheric nitrogen.

Pure synthetic $CaCO_3$ (without acetanilide) had peaks similar to empty tin cups (pure $CaCO_3$ yielded 0.43 ± 0.03 Vs (n = 4, see Supporting Information); empty tin cup = 0.49 Vs) and therefore did not contribute much to the calculated delta values. The acetanilide standard had a $\delta^{15}N$ value of −2.12 ± 0.13‰ (n = 5; area = 14 to 38 Vs) when it was run without synthetic $CaCO_3$ and was −2.02 ± 0.11‰ (n = 14; area = 11 to 39 Vs for data above 15 μg N; data between 5 and 15 μg N = −2.11 ± 0.36‰, n = 7) when it was run with 98.4 to 66.8% $CaCO_3$ (Fig. 1; see Supporting Information). These values are not significantly different ($p = 0.080$). In addition, during a preliminary trial, we ran 0.4 mg of the IAEA N1 ammonium sulfate ($(NH_4)_2SO_4$) standard (certified $\delta^{15}N$ value = 0.4 ± 0.2‰) in 15.72 mg $CaCO_3$ and found no offset from N1 standards run without $CaCO_3$ ($\delta^{15}N$ = 0.33‰; equivalent to 84.8 μg N or 0.5% N). Our results show that samples with as little as 20 μg N (or 1.6% N) can provide accurate $\delta^{15}N$ values (Fig. 1). Prior acidification is not required to eliminate the carbonate matrix to produce accurate results, as has been previously reported.[14] It should be noted that mollusks with very low organic matrix in their shells may require a pre-concentration step to reduce the poorer precision of small samples. However, considering the large fractionations associated with nitrogen isotopes in nature, even samples between 5 and 15 μg N provided reasonable data (1σ = 0.36). There is also an upper limit to the amount of shell material that can be loaded into the EA, but this was not evaluated here.

This method is robust because calcium carbonate completely decomposes around 825°C and the flash combustion in the EA was around 1020°C; therefore, all N should be released from the matrix and carried to the IRMS. Moreover, previous studies have used an EA-IRMS system to combust carbonates for $\delta^{13}C$ analysis.[36] It is clear from the traces in Fig. 2 that the narrow and near-symmetrical peak shapes are similar for both shell carbonate and synthetic mixtures, which suggests that both matrices are reacting similarly in the EA-IRMS. We therefore argue that it is possible to measure $\delta^{15}N$ values of $CaCO_3$-rich samples by simple combustion,

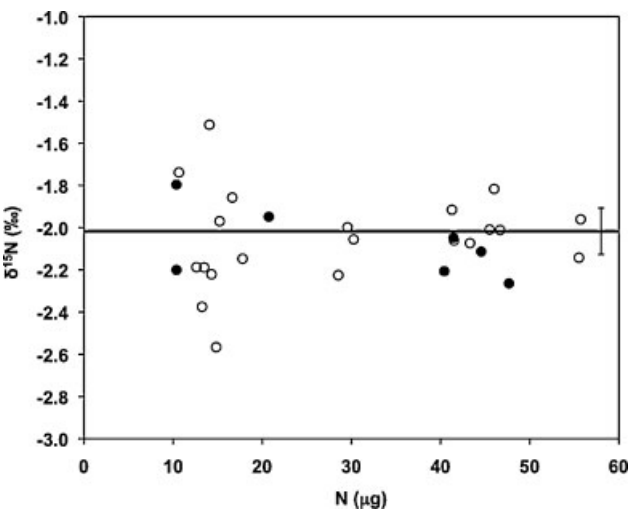

Figure 1. $\delta^{15}N$ values for acetanilide mixed with 66.8 to 98.4 weight % synthetic $CaCO_3$ powder (open symbols) and pure acetanilide (closed symbols). The solid line represents the mean value of −2.02‰ (1σ = 0.11; n = 14) for data above 15 μg N (the average for data between 5 and 15 μg N is −2.11‰, 1σ = 0.36, n = 7). The error bar represents the 1σ of ±0.11‰.

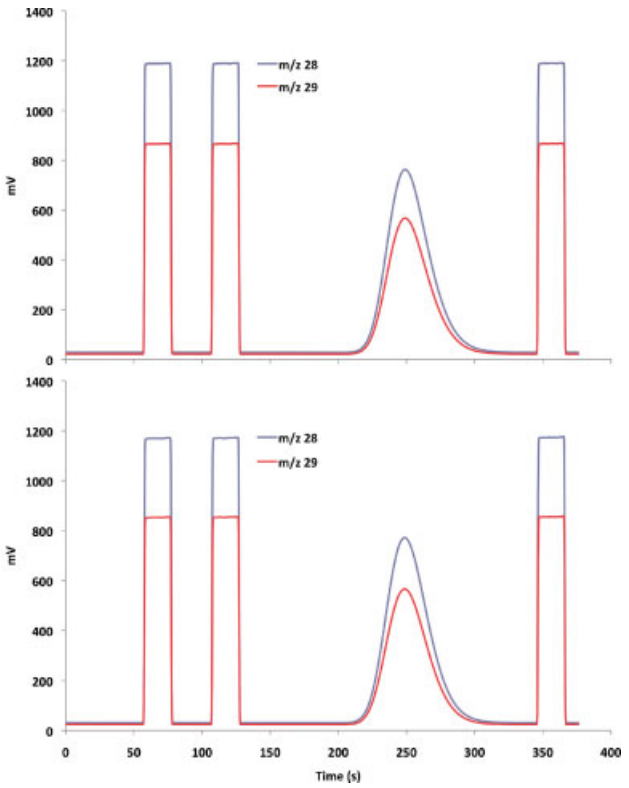

Figure 2. Example IRMS responses of combusted shell material (A; 17.54 mg) and synthetic $CaCO_3$/acetanilide mixture (B; 8.36 mg $CaCO_3$ and 0.44 mg acetanilide; 46 μg N or 0.5% N). The raw traces for both masses are very similar between the two sample types (e.g., peak centers both at 249 s). The three rectangular peaks (peaks 1, 2 and 4) are the reference gas peaks supplied by the Conflo interface. The upper trace is *m/z* 28 and the lower is *m/z* 29.

avoiding any possible adverse effects[22,23] and the increased sample preparation time of the acidification step.

In order to reconstruct historical environmental $\delta^{15}N$ values, we need to compare $\delta^{15}N$ values from shell organic matrix with those from soft tissues to determine if an offset needs to be applied. This will allow the application of our knowledge of tissue nitrogen dynamics to be applied to shells, such as the 3 to 4‰ trophic enrichment associated with $\delta^{15}N$ values in animals (see O'Donnell *et al.*[12]). The three modern shells for which we measured both shell and soft tissues show that shell organic matter had on average 2.2 ‰ higher $\delta^{15}N$ values than mantle tissue ($\Delta_{tissue-shell} = -2.2 \pm 1.5$‰). Between individuals, shell organic matter $\delta^{15}N$ values varied by only 0.2‰, while mantle tissue $\delta^{15}N$ values varied by 3‰ (Table 2). This is probably due to the fact that the mantle tissue is subject to metabolic turnover and is thus representative for a specific time window, see e.g., Paulet *et al.*[37], while the shell samples averaged at least 1 year of growth. This makes comparing soft tissues with shell organic matrix difficult. However, as shown in Delong and Thorp,[31] tissues with slower turnover rates, such as the adductor muscle, are better for comparisons with metabolically inactive shells. Most previous studies that report differences between skeletal $\delta^{15}N$ and soft tissue $\delta^{15}N$ (i.e. Δ values) do not take the different amounts of time being averaged into consideration. Moreover, many studies compare whole body tissue $\delta^{15}N$ data to shell data (see Table 3) while it is known that different organs can have quite different $\delta^{15}N$ values, sometimes as much as 5‰ in the same animal.[8] This may explain why $\Delta_{tissue-shell}$ values for the same species of clam (*Mercenaria mercenaria*) range from 0.2 to 2.4‰, see O'Donnell *et al.*[12] (Table 3). Soft tissue $\delta^{15}N$ data from *M. edulis* specimens collected at three different periods in 2002 from Knokke show significant changes throughout the year (March = 12.9 ± 0.2‰, n = 3; May = 10.6 ± 0.6‰, n = 8; Sept. = 12.3 ± 0.5‰, n = 14; Gillikin, unpublished data), which would be averaged in the shell samples we analyzed. Taking the average of these 25 soft tissue data (11.9 ± 1.0‰) results in a $\Delta_{tissue-shell}$ value of −1.5 ± 1.0‰. In the future it is important to compare tissues and shells that represent the same time period.

To extend $\delta^{15}N$ values back in time, museum specimens have the largest potential to provide unaltered $\delta^{15}N$ values. Ethanol-preserved shells had significantly different $\delta^{15}N$ values from dry-stored specimens, being ^{15}N depleted by 5.2 ± 2.3‰ ($p = 0.018$; Fig. 3). There was no significant difference in $\delta^{15}N$ values between the dry-stored specimens of 1936 and 1938 (the difference between storage methods when shells from these years are combined is 5.9 ± 2.2‰ ($p < 0.001$)). The difference between dry and wet preserved specimens could be due to bacterial decay of dry stored specimens (or some other processes altering the dry shells) thereby enriching the organic matrix in ^{15}N, or due to the ethanol altering the $\delta^{15}N$ value of the shell organic matrix. While we cannot prove either process caused the shift, we suggest that the ethanol-preserved shells are altered and the dry-stored shells are not. We hypothesize that the soft tissues, with abundant N, leached ^{14}N into the ethanol solution (the ethanol residue was about 0.4‰ lighter than the tissues; Fig. 3), which was then taken up into the shell shells soaking in this solution for more than 70 years. It is possible that the shell organic matrix incorporated ^{14}N more readily thereby making the shells more negative than the ethanol residue. Previous studies have found that preserved tissues may shift toward the isotopic value of the preservative, see Sarakinos *et al.*,[30] and references cited therein. Moreover, dry museum storage is generally considered to preserve original $\delta^{15}N$

Table 2. Shell and mantle tissue $\delta^{15}N$ values for three shells from Knokke, Belgium

Name	Collection date	Shell $\delta^{15}N$ (‰)	Mantle tissue $\delta^{15}N$ (‰)	$\Delta_{tissue-shell}$ (‰)
K2	17 March 02	13.4	12.7	−0.7
K7	3 May 02	13.3	10.9	−2.3
K10	3 May 02	13.5	9.8	−3.7

Table 3. Summary of studies reporting $\delta^{15}N$ values in the organic matrix of bivalve shells and fish otoliths. Positive Δ values represent lighter values in shell compared to soft tissues (modified after Carmichael & Kovacs[39])

Species	$\Delta_{\text{tissue–shell/otolith}}$ (‰)	Soft tissue	Age class	$CaCO_3$ removal method	Source
Ruditapes philippinarum	1.1 ± 0.4*	Whole tissues	NR	Acidification	Watanabe *et al.*[13]
Mercenaria mercenaria	2.4 ± 0.3	Whole tissues	Juveniles, adults	Acidification	Carmichael *et al.*[14]
Mercenaria mercenaria	1.0 ± 0.8*	Adductor muscle	Juveniles, adults	Acidification	O'Donnell *et al.*[12]
Mercenaria mercenaria	0.7 ± 0.8*	Foot	Juveniles, adults	Acidification	O'Donnell *et al.*[12]
Mercenaria mercenaria	0.2 ± 0.7*	Mantle	Juveniles, adults	Acidification	O'Donnell *et al.*[12]
Arctica islandica	2.7*	Whole tissues	NR	Dialysis, acidification	LeBlanc[15]
Mytilus edulis	−0.1 ± 0.2	Whole tissues	NR	Dialysis, acidification	LeBlanc[15]
Totoaba macdonaldi (fish)	0.76	Muscle	Juveniles	Simple combustion	Rowell *et al.*[17]
Several freshwater fish species	1.1	Muscle	Adults	Simple combustion	Vandermyde and Whitledge[18]
Mytilus edulis	−2.2 to −1.5**	Mantle	Adults	Simple combustion	This study

* Averages calculated by Carmichael & Kovacs.[39]

** Considering average soft tissue values of animals collected at various times throughout the year.

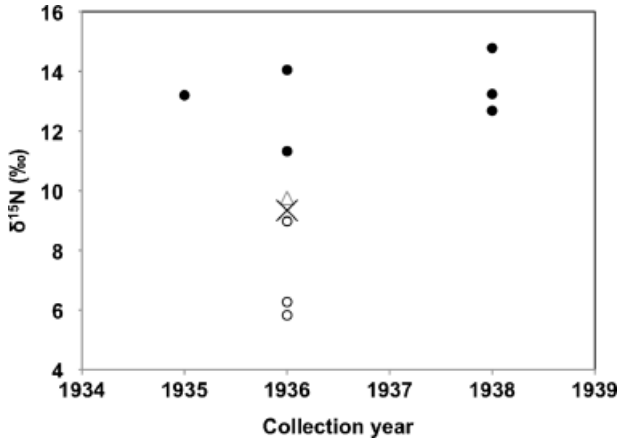

Figure 3. Shell organic matrix $\delta^{15}N$ values for dry-stored (filled circles) and ethanol-preserved (open circles) *M. edulis* shells. Mantle tissue $\delta^{15}N$ values for the ethanol-preserved specimens are also shown (pooled tissues, open triangle), as is the residue from a dried aliquot of the ethanol they were preserved in (X symbol). Ethanol-preserved shells are depleted in ^{15}N by 5.2 ± 2.3‰ on average compared to dry-stored shells ($p = 0.018$). Note that there are two data at 11.3‰ for the filled 1936 circles.

values in organic matter, e.g. Delong *et al.*[31] This suggests that ethanol-preserved shells without tissues may not be as altered as the shells analyzed here.

Due to the scarcity of these old museum specimens we could only analyze a limited number of shells. More work on these long-term stored samples is desirable to determine if this ^{15}N depletion is caused by wet or dry storage and also if it occurs in other bivalve tissues and animal taxa, and with other liquid preservation methods. Until the precise effect of ethanol preservation on shell samples is known, $\delta^{15}N$ values of museum specimens should be treated with caution. This also highlights the fact that detailed studies on the effect of diagenesis on $\delta^{15}N$ values in shell organic matrix are needed before this proxy can confidently be applied to archeological or geological specimens.

In summary, simple combustion of bivalve shells is a robust method for analyzing $\delta^{15}N$ values of *Mytilus* shell organic matter. Direct calculations of differences between shell and soft tissue $\delta^{15}N$ values are difficult due to differences in time scales over which the isotopic signal is integrated in these different substrates. The large sample size needed for shell material results in significant time-averging, while tissues can average weeks to months, e.g. Paulet *et al.*[37] and Fukumori *et al.*[38] Different mollusk species probably have different amounts of organic matter and thus %N; some concentration method (e.g., acid dissolution of shell carbonate) may be required for species with very low %N in their shells when very precise $\delta^{15}N$ data are needed. Moreover, although $\delta^{15}N$ values of shell organic matter have the potential to provide a wealth of information, more information regarding the effects of long-term storage and diagenesis needs to be investigated.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

We thank Thierry Backeljau at the Royal Belgian Institute of Natural Sciences and Frank Wesselingh at the Dutch National Museum of Natural History Naturalis for providing historical shell material. We also thank Michael Korntheuer for assistance with running the IRMS and for help with method development, David Dettman for useful discussions on combusting shells for $\delta^{15}N$ analysis, and Steven Bouillon for help with Isodat. Christopher Romanek, Alan Wanamaker, and two anonymous reviewers provided comments that significantly improved an earlier version of this manuscript. Funding was provided by the Belgian Federal Science Policy Office, Brussels, Belgium (CALMARS II, contract SD/CS/02 A and B).

Emma A. A. Versteegh[1†], *David P. Gillikin*[2]* *and Frank Dehairs*[1]

[1]Department of Analytical and Environmental Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

[2]Department of Geology, Union College, 807 Union St., Schenectady, NY 12308, USA

**Correspondence to: D. P. Gillikin, Department of Geology, Union College, 807 Union St., Schenectady, NY 12308, USA.*
E-mail: gillikid@union.edu

[†]*Current address: Department of Geography and Environmental Science, University of Reading, Reading RG6 6AB, UK.*

REFERENCES

[1] M. J. Deniro, S. Epstein. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* **1981**, *45*, 341.
[2] M. J. Vander Zanden, J. B. Rasmussen. Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: Implications for aquatic food web studies. *Limnol. Oceanogr.* **2001**, *46*, 2061.
[3] M. J. Vander Zanden, J. B. Rasmussen. Primary consumer $\delta^{13}C$ and $\delta^{15}N$ and the trophic position of aquatic consumers. *Ecology* **1999**, *80*, 1395.
[4] G. Cabana, J. B. Rasmussen. Comparison of aquatic food chains using nitrogen isotopes. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 10844.
[5] S. Hansson, *et al*. The stable nitrogen isotope ratio as a marker of food-web interactions and fish migration. *Ecology* **1997**, *78*, 2249.
[6] J. McClelland, W. I. Valiela, R. H. Michener. Nitrogen-stable isotope signatures in estuarine food webs: A record of increasing urbanization in coastal watersheds. *Limnol. Oceanogr.* **1997**, *42*, 930.
[7] R. H. Carmichael, B. Annett, I. Valiela. Nitrogen loading to Pleasant Bay, Cape Cod: application of models and stable isotopes to detect incipient nutrient enrichment of estuaries. *Mar. Pollut. Bull.* **2004**, *48*, 137.
[8] A. Lorrain, *et al*. Differential $\delta^{13}C$ and $\delta^{15}N$ signatures among scallop tissues: implications for ecology and physiology. *J. Exp. Mar. Biol. Ecol.* **2002**, *275*, 47.
[9] B. Fry. Conservative mixing of stable isotopes across estuarine salinity gradients: A conceptual framework for monitoring watershed influences on downstream fisheries production. *Estuaries and Coasts* **2002**, *25*, 264.
[10] R. A. McKinney, J. L. Lake, M. A. Charpentier, S. Ryba. Using mussel isotope ratios to assess anthropogenic nitrogen inputs to freshwater ecosystems. *Environ. Monit. Assess.* **2002**, *74*, 167.
[11] R. A. McKinney, W. G. Nelson, M. A. Charpentier, C. Wigand. Ribbed mussel nitrogen isotope signatures reflect nitrogen sources in coastal salt marshes. *Ecol. Appl.* **2001**, *11*, 203.
[12] T. H. O'Donnell, *et al*. Analysis of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ in organic matter from the biominerals of modern and fossil *Mercenaria* spp. *Org. Geochem.* **2003**, *34*, 165.
[13] S. Watanabe, M. Kodama, M. Fukuda. Nitrogen stable isotope ratio in the manila clam, *Ruditapes philippinarum*, reflects eutrophication levels in tidal flats. *Mar. Pollut. Bull.* **2009**, *58*, 1447.
[14] R. H. Carmichael, T. Hattenrath, I. Valiela, R. H. Michener. Nitrogen stable isotopes in the shell of *Mercenaria mercenaria* trace wastewater inputs from watersheds to estuarine ecosystems. *Aquatic Biol.* **2008**, *4*, 99.
[15] C. LeBlanc. Terrestrial input to estuarine bivalves as measured by multiple stable isotopes tracers. *PhD thesis, McMaster, Montreal, Canada,* **1989**.
[16] B. Williams, A. G. Grottoli. Stable nitrogen and carbon isotope ($\delta^{15}N$ and $\delta^{13}C$) variability in shallow tropical Pacific soft coral and black coral taxa and implications for paleoceanographic reconstructions. *Geochim. Cosmochim. Acta* **2010**, *74*, 5280.
[17] K. Rowell, D. Dettman, R. Dietz. Nitrogen isotopes in otoliths reconstruct ancient trophic position. *Environ. Biol. Fishes* **2010**, 1.
[18] J. M. Vandermyde, G. W. Whitledge. Otolith $\delta^{15}N$ distinguishes fish from forested and agricultural streams in southern Illinois. *J. Freshwater Ecol.* **2008**, *23*, 333.
[19] R. D. Dietz. Nitrogen isotopes in bivalve shells from the Colorado River estuary: Evaluating a potential proxy for changes in riverine nutrient delivery, *MSc thesis*, University of Arizona, **2008**.
[20] B. Fry. Food web structure on Georges Bank from stable C, N, and S isotopic compositions. *Limnol. Oceanogr.* **1988**, *33*, 1182.
[21] S. Bouillon, N. Koedam, A. V. Raman, F. Dehairs. Primary producers sustaining macro-invertebrate communities in intertidal mangrove forests. *Oecologia* **2002**, *130*, 441.
[22] M. Mateo, O. Serrano, L. Serrano, R. Michener. Effects of sample preparation on stable isotope ratios of carbon and nitrogen in marine invertebrates: implications for food web studies using stable isotopes. *Oecologia* **2008**, *157*, 105.
[23] U. Jacob, *et al*. Stable isotope food web studies: a case for standardized sample treatment. *Mar. Ecol. Prog. Ser.* **2005**, *287*, 251.
[24] O. Serrano, *et al*. Acid washing effect on elemental and isotopic composition of whole beach arthropods: Implications for food web studies using stable isotopes. *Acta Oecol.* **2008**, *34*, 89.
[25] J. S. S. Ng, T.-C. Wai, G. A. Williams. The effects of acidification on the stable isotope signatures of marine algae and molluscs. *Mar. Chem.* **2007**, *103*, 97.
[26] K. L. Bosley, S. C. Wainright. Effects of preservatives and acidification on the stable isotope ratios (^{15}N:^{14}N, ^{13}C:^{12}C) of two species of marine animals. *Can. J. Fish. Aquat. Sci.* **1999**, *56*, 2181.
[27] J. Syväranta, *et al*. Evaluating the utility of stable isotope analyses of archived freshwater sample materials. *Hydrobiologia* **2008**, *600*, 121.
[28] D. A. Arrington, K. O. Winemiller. Preservation effects on stable isotope analysis of fish muscle. *Trans. Am. Fisheries Soc.* **2002**, *131*, 337.
[29] M. S. Edwards, T. F. Turner, Z. D. Sharp. Short- and long-term effects of fixation and preservation on stable isotope values ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) of fluid-preserved museum specimens. *Copeia* **2002**, 1106.
[30] H. C. Sarakinos, M. L. Johnson, M. J. Vander Zanden. A synthesis of tissue-preservation effects on carbon and nitrogen stable isotope signatures. *Can. J. Zool.* **2002**, *80*, 381.
[31] M. D. Delong, J. H. Thorp. Mollusc shell periostracum as an alternative to tissue in isotopic studies. *Limnol. Oceanogr. Methods* **2009**, *7*, 436.

[32] S. Kaehler, E. A. Pakhomov. Effects of storage and preservation on the $\delta^{13}C$ and $\delta^{15}N$ signatures of selected marine organisms. *Mar. Ecol. Prog. Ser.* **2001**, *219*, 299.
[33] S. Carabel, P. Verísimo, J. Freire. Effects of preservatives on stable isotope analyses of four marine species. *Estuar. Coast. Shelf. Sci.* **2009**, *82*, 348.
[34] D. P. Gillikin, *et al*. Barium uptake into the shells of the common mussel (*Mytilus edulis*) and the potential for estuarine paleo-chemistry reconstruction. *Geochim. Cosmochim. Acta* **2006**, *70*, 395.
[35] J. K. Böhlke, T. B. Coplen, *Interlaboratory Comparison of Reference Materials for Nitrogen-Isotope-Ratio Measurements*, IAEA, Vienna, **1995**.
[36] H. Qi, T. B. Coplen, H. Geilmann, W. A. Brand, J. K. Böhlke. Two new organic reference materials for $\delta^{13}C$ and $\delta^{15}N$ measurements and a new value for the $\delta^{13}C$ of NBS 22 oil. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2483.
[37] Y.-M. Paulet, A. Lorrain, J. Richard, S. Pouvreau. Experimental shift in diet $\delta^{13}C$: A potential tool for ecophysiological studies in marine bivalves. *Org. Geochem.* **2006**, *37*, 1359.
[38] K. Fukumori, *et al*. Bivalve tissue as a carbon and nitrogen isotope baseline indicator in coastal ecosystems. *Estuar. Coast. Shelf. Sci.* **2008**, *79*, 45.
[39] R. H. Carmichael, C. J. Kovacs, Comment on Watanabe et al. (2009). *Mar. Pollut. Bull.* **2010**, *60*, 314.

