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LIMNOLOGY
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OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 6, 2008, 12–15
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Effects of acidification in multiple stable isotope analyses

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Abstract

The effect of in situ acidification on the stable isotope ratios of carbon and nitrogen was tested in several invertebrates living in an eelgrass system. Dried and ground samples of individuals were weighted in silver cups and treated in situ with 10% HCl. Control samples were measured without acidification. This treatment to remove inorganic carbon significantly decreased the $\delta^{13}C$ values. The $\delta^{15}N$ values were not affected by this method of acidification. In contrast to the acid-washing method the tested procedure seems suitable to remove inorganic carbon in small invertebrate species.

Stable isotope analysis has been proven to be an important tool to understand food web dynamics in aquatic ecosystems. Stable carbon isotopic signatures are commonly used to recognize and quantify potential food sources (Stephenson et al. 1986, Moncreiff and Sullivan 2001, Fredriksen 2003). However, samples, which contain nondietary carbon with deviating $\delta^{13}C$ values, can cause problems in this methodological approach. Especially sediment samples, carbonate-encrusted algae, and mollusks and crustaceans, which are too small to dissect muscle material, can comprise relevant amounts of inorganic carbon. The shell of mollusks is formed by calcium carbonate and the basically chitinous body wall of crustaceans is usually reinforced with calcium carbonate to generate a rigid exoskeleton. Earlier stable isotope studies used different variations of the acid washing method to remove nondietary carbon (Fry et al. 1982, Peterson and Howarth 1987, Sullivan and Moncreiff 1990). Bunn et al. (1995) found that this method significantly influences the $\delta^{15}N$ values of shrimp samples. Nevertheless, this method is used in many recent benthic studies with several variations concerning acid concentration and duration of the bathing in acid (Marguillier et al. 1997, Kharlamenko et al. 2001, Moncreiff and Sullivan 2001, Fredriksen 2003, Kang et al. 2003). Some studies totally dispense with acidification (Fourqurean et al. 1997, Jennings et al. 1997, Loneragan et al. 1997, Connolly et al. 1995) and a few studies used an in situ acidification procedure (Deegan and Garritt 1997, Herman et al. 2000). Here, the pulverized samples were acidified with a small amount of relative high concentrated HCl. To investigate the effect of this method on stable isotope ratios, we analyzed several invertebrate species collected in an eelgrass bed in the Kiel Fjord, Western Baltic Sea.

Materials and procedures

The study included eight species: isopod *Idotea baltica* (analyzed were adult [15 mm] and juvenile specimen [3-5 mm]), amphipods *Gammarus oceanicus* (10 mm) and *Amphithoe rubricata* (13 mm), shrimps *Praunus flexuosus* (25 mm) and *Crangon crangon* (30-35 mm), and gastropods *Rissoa membranacea* (5 mm) and *Lacuna vincta* (5 mm). All animals were kept alive overnight in filtered sea water to clear their guts (Hobson and Welch 1992), rinsed with distilled water and dried to constant weight (60°C, 48 h). Ten individuals were ground with an agate mortar and pestle as fine as possible and then stored in clean airtight plastic vials until further processing. In the case of the small gastropods, ten individuals were pooled to obtain sufficient material for analysis. Two splits of each sample (0.4-0.8 mg depending on the species) were weighted into silver cups. One sample was acidified with 0.2 µL 10% HCL, the other served as control. The samples were dried for 1 h at 60°C, and then another 0.2 µL 10% HCl was added to confirm the complete removal of inorganic carbon. The samples were dried again for 12 h at 60°C to remove hydrochloric acids to avoid contamination of the CN-analyzer. Directly afterward, the cups were closed, compacted, and analyzed.

The amount of HCl necessary to remove all inorganic carbon was tested beforehand. Three samples of each species were acidified with 0.2 µL and 0.4 µL 10% HCl, respectively. We found no significant differences between these treatments. Thus, the used amount of HCl should be adequate to remove the inorganic carbon completely. The acid was added sequentially to avoid sample loss by effervescence. We choose a relatively weak HCl solution, because higher concentrations of acid improve the reproducibility (Nieuwenhuize et al. 1994), but can bias the $\delta^{15}N$ values (Kennedy et al. 2005).

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Acknowledgment
This analysis was funded by the German Research Foundation (SO 145/20).

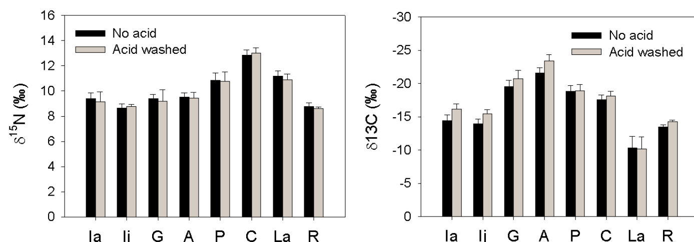

Figure 1. Mean stable isotope composition (± SD) of δ^{15}N and δ^{13}C ratios in no acid and acid treatments (Ia = adult *I.baltica*, Ij = juvenile *I. baltica*, G = *G. oceanicus*, A = *A. rubricata*, *P* = *P. flexuosus*, C = *C. crangon*, L = *L. vincta* and R = *R. membranacea*)

All samples were combusted in a CN-analyzer (Thermo Electron, NA 1110) connected to a Finnigan Delta plus mass spectrometer. δ^{15}N and δ^{13}C ratios were calculated as

$$\delta X (‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X = ^{15}N or ^{13}C and R = ^{15}N/^{14}N or ^{13}C/^{12}C. Pure N_2 and CO_2 gas were used as primary standard and calibrated against IAEA reference standards (N1, N2, N3, NBS22, and USGS24). Acetanilide was used as internal standard after every sixth sample. The overall analytical precision was ± 0.1‰ for δ^{15}N and δ^{13}C.

The differences between no acid and acid-treated samples were tested with paired *t* tests for each species.

Assessment and discussion—All species showed a decrease in δ^{13}C values (Figure 1) ranging from 0.12‰ in *Praunus flexuosus* to 1.77‰ in *Amphitoe rubricata*. The difference between no acid and acid treatment was not significant for the small gastropod *Lacuna vincta* and the crustacean species *Praunus flexuosus* and *Crangon crangon* (Table 1). The acid treatment had no significant effect on the δ^{15}N values (Figure 1). No significant difference in variation between individuals in the acid-treated samples was found for δ^{13}C values. The variation in δ^{15}N values increased from mean 4.0% to 5.7%, but the change was not significant (Table 1).

The effect of acidification on δ^{13}C and δ^{15}N values remains inconclusive so far. Bunn and colleagues' study (1995) on the effects of acid washing resulted in statistically and ecologically important changes in δ^{13}C and δ^{15}N values in shrimp and seagrass. Additionally, the variation among individuals broadened in δ^{13}C and δ^{15}N values, leading to a decrease in statistical power for testing differences between samples. Carabel et al. (2006) observed a decrease in δ^{13}C values of SOM, SPOM, plankton, and in two swimming crabs, whereas a decrease of δ^{15}N values was only found for SOM. Changes in δ^{15}N values after acidification were shown for fish muscle by Pinnegar and Polunin (1999). Jacob et al (2005) demonstrated a strong effect of acidification on δ^{13}C values in 29 Antarctic invertebrate and fish species, whereas the changes in δ^{15}N values were negligible. Further studies found no significant effect on carbon and/or nitrogen stable isotopes of plankton, invertebrate, and fish species (Bosley and Wainright 1999, Chanton and Lewis 1999, Grey et al. 2001, Carabel et al. 2006).

The effect of acidification on carbon depends mainly on the sample carbonate content (Jacob et al. 2005). Sediment samples and species with shells or exoskeletons containing inorganic carbon must be acidified to be able to compare the data.

The effects of acidification on nitrogen seem to depend on the applied procedure. Our results support the position that the changes in δ^{15}N values are primarily caused by the rinsing of the acidified sample (Bunn et al. 1995, Bosley and Wainright 1999, Jacob et al. 2005).

Table 1. Mean stable isotope ratios (± SD) and the results of paired *t* test between no acid and acid-washed samples

Species	No acid δ^{15}N	Acid-washed δ^{15}N	*t*	*P*
I. baltica	9.38 ± 0.48	9.15 ± 0.77	0.815	0.426
I. baltica, juv.	8.65 ± 0.33	8.78 ± 0.15	–1.081	0.294
G. oceanicus	9.41 ± 0.33	9.19 ± 0.9	0.730	0.475
A. rubricata	9.52 ± 0.31	9.46 ± 0.42	0.406	0.690
P. flexuosus	10.86 ± 0.57	10.78 ± 0.74	0.270	0.790
C. crangon	12.84 ± 0.41	13.01 ± 0.43	–0.861	0.401
L. vincta	11.18 ± 0.44	10.91 ± 0.45	0.730	0.506
R. membranacea	8.77 ± 0.30	8.61 ± 0.14	0.875	0.431
Species	**No acid δ^{13}C**	**Acid washed δ^{13}C**	***t***	***P***
I.baltica	–14.44 ± 0.87	–16.17 ± 0.80	4.63	<0.001
I.baltica, juv.	–13.98 ± 0.66	–15.48 ± 0.60	5.31	<0.001
G.oceanicus	–19.52 ± 1.00	–20.70 ± 1.30	2.263	0.036
A.rubricata	–21.57 ± 0.84	–23.34 ± 0.92	4.635	<0.001
P.flexuosus	–18.82 ± 0.90	–18.94 ± 0.91	0.287	0.777
C.crangon	–17.56 ± 0.70	–18.14 ± 0.73	1.827	0.084
L. vincta	–10.32 ± 1.77	–10.19 ± 1.78	–0.089	0.933
R. membranacea	–13.46 ± 0.36	–14.27 ± 0.24	3.227	0.032

Goering et al. (1990) also found changes in HCl-treated samples and suggested that the different loss of compounds containing nitrogen caused these results. These results implicated that different organic nitrogen compounds had varying $\delta^{15}N$ values. The presumed leaching of small molecules while rinsing the samples is supported by the fact that grounding increased the effect of acid washing (Bunn et al. 1995).

The vapor phase acidification is another method that prevents the loss of nitrogen compounds. However, this method should not be applied to samples containing high amounts of carbonate (e.g., gastropod shells) because the removal of carbonates may be incomplete (Hedges and Stern 1984, Schubert and Nielsen 2000).

The strength and variety of the used acid can also influence the effects of acidification. Kennedy et al. (2005) observed that H_2SO_3, and H_3PO_4 caused heavier $\delta^{13}C$ values compared to HCl solutions, probably resulting from incomplete removal of carbonate. Furthermore, the $\delta^{15}N$ values were significantly affected under 6 M HCI, H_2SO_3, and H_3PO_4 treatments.

The in situ acidification method with weak HCL without rinsing evades these problems and can be efficiently applied to samples, where the removal of nondietary carbon is absolutely necessary. In larger animals it is possible to discard parts containing calcium carbonate (e.g shells in mollusks and exoskeletons in crustacean), but small species are usually crushed in total. These samples can contain significant amount of nondietary carbon that can influence the results on potential food sources. Furthermore, the nitrogen in the exoskeleton originates from the diet and may be relevant in determining consumer food sources. The "champagne test" can be used to test for relevant amounts of inorganic carbon in samples. A small amount of the sample is dropped in a Petri dish, which contains 10% HCl. If carbonate is present, effervescence will be visible.

Comments

Our results suggest that the used in situ acidification method can be applied to eliminate nondietary carbon without significantly influencing the chemical composition of the studied samples. Nevertheless, we recommend that samples only be acidified if absolutely necessary.

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Submitted 15 December 2006
Revised 1 August 2007
Accepted 8 October 2007