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## Effects of acid washing on stable isotope ratios of C and N in penaeid shrimp and seagrass: Implications for food-web studies using multiple stable isotopes

**Abstract**—We investigated the effects of acid washing on the carbon and nitrogen composition and stable isotope ratios of C and N in shrimp (*Metapenaeus* spp.) and seagrass (*Enhalus acoroides*). Acid washing did not affect the mean  $\delta^{13}\text{C}$  ratios for juvenile *Metapenaeus moyebi* and resulted in only an ecologically insignificant change (0.3‰) in mean  $\delta^{13}\text{C}$  ratios for larger *Metapenaeus bennettiae*. In contrast, acid washing increased the mean  $\delta^{15}\text{N}$  signatures of shrimp tissue (~3‰) and decreased that of seagrass (~1.8‰) to a degree that may confound the interpretation of food webs. The increase in %C and %N in both shrimp and seagrass after acid washing suggests that the changes in isotope ratios are due to loss of molecules comparatively low in C and N. Treating samples by acid washing also resulted in an increase in the variation among individuals for both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , which would lead to a loss of statistical power for testing differences between species, sites, or seasons.

Stable isotope tracing is becoming an increasingly important tool in studies of aquatic food webs (Peterson and Fry 1987; Preston 1992). Although most research has centered on the use of stable isotopes of carbon (Rounick and Winterbourn 1986; Peterson and Fry 1987), the isotopes of other elements, particularly nitrogen and sulfur, are being measured more frequently in food-web studies (Peterson and Fry 1987; Hesslein et al. 1991).

With the advent of new technology, such as continuous flow isotope ratio mass spectrometry (CF-IRMS), it is now possible to obtain isotopic ratios of more than one element from individual samples (Fry et al. 1992; Preston 1992). However, many of the techniques for sample preparation described in previous studies were developed for  $\delta^{13}\text{C}$  alone and may not be applicable to studies where multiple stable isotope ratios are measured simultaneously.

For example, samples of sediments, molluscs, crustaceans, and fish scales have been acid washed with dilute HCl to remove traces of nondietary carbonate (Rau et al. 1983; Jackson et al. 1986). Acid washing has also been used to separate epiphytes from their host macrophytes (Kitting et al. 1984). Carbonates continue to be removed from animal tissue by acid washing in the preparation of samples for multiple stable isotope analyses (Hobson and Welch 1992).

To examine the effects of sample preparation on stable isotope ratios, we investigated the influence of acid washing on  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and the C and N composition of penaeid shrimp and seagrass.

Samples of adult shrimp (*Metapenaeus bennettiae*) ranging from ~20 to 30 mm in carapace length from

Moreton Bay (27°30'S, 153°15'E, collected in November 1990), southeast Queensland, were analyzed. The exoskeletons and digestive tracts of five shrimp were removed and the tail muscle tissue dried to constant weight (60°C for 36–48 h). The muscle tissue was cut longitudinally in half; half was ground and analyzed (no-acid treatment), and the other half was bathed in 0.1 N HCl for 1 h at room temperature and then rinsed in distilled water and dried and ground (acid-washed treatment). Three subsamples of muscle tissue from each half of shrimp tail muscle were oxidized in a Roboprep CN biological sample converter (Dumas combustion) and the resultant  $\text{CO}_2$  and  $\text{N}_2$  analyzed with a CF-IRMS (Europa Tracermass). Ratios of  $^{13}\text{C}:^{12}\text{C}$  and  $^{15}\text{N}:^{14}\text{N}$  were expressed as the relative per mil difference between the sample and conventional standards (PDB carbonate and  $\text{N}_2$  in air) as follows:

$$\delta X = (R_{\text{sample}}/R_{\text{std}} - 1) \times 1,000(\text{‰}).$$

$X$  is  $^{13}\text{C}$  or  $^{15}\text{N}$  and  $R$  is  $^{13}\text{C}:^{12}\text{C}$  or  $^{15}\text{N}:^{14}\text{N}$ .

Mean values were calculated for the three subsamples for each half of the muscle tissue. Differences between the no-acid and acid-washed treatments were tested with paired  $t$ -tests.

Although acid-washed shrimp were significantly depleted in  $^{13}\text{C}$ , it was only by 0.3‰ (mean  $\delta^{13}\text{C} \pm 1$  SE for no acid =  $-16.2 \pm 0.08\text{‰}$ ; mean for acid washed =  $-16.5 \pm 0.10\text{‰}$ ,  $t_4 = 12.3$ ,  $P < 0.001$ ). Acid-washed shrimp were, however, markedly enriched in  $^{15}\text{N}$  by 2.9‰ (mean  $\delta^{15}\text{N}$  for no acid =  $9.0 \pm 0.39\text{‰}$ ; mean for acid washed =  $11.9 \pm 0.41\text{‰}$ ,  $t_4 = 4.2$ ,  $P = 0.014$ ).

We further investigated the effects of acid washing on shrimp (mainly *Metapenaeus moyebi*, 6–10 mm in carapace length) collected from a tropical seagrass bed in the Embley River estuary (12°40'S, 141°50'E), northern Australia, in November 1991. The effects of acid washing (bathed in 0.1 N HCl at room temperature for 1 h, then rinsed in distilled water) and removing the exoskeleton from the tail muscle tissue of the shrimp on the composition of C and N (%) and the stable isotope ratios of C and N were tested in a two-way factorial design. Ten shrimp were used in each treatment. The factors of acid and exoskeleton each had two treatment levels: no acid and acid washed; muscle only and muscle+exoskeleton. Digestive tracts were removed from the tail muscle in each treatment. ANOVAs were used to test the significance of the main factors and their interactions. An additional treatment, in which the exoskeleton and digestive tract were removed and muscle tissue was dried and ground, then acid washed and rinsed in distilled water (four times) before drying to constant weight, was also

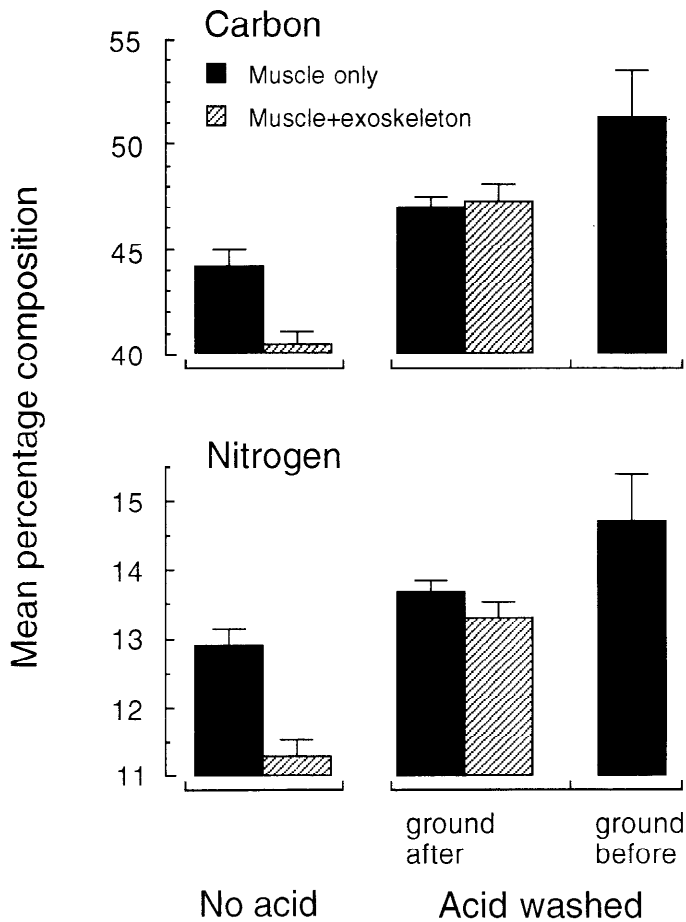


Fig. 1. Mean percentage composition ( $\pm 1$  SE) of C and N in tail muscle tissue of *Metapenaeus moyebi* for different treatments.

tested on 10 shrimp. Means from this ground-before-acid treatment were compared with those from the other muscle-only treatments by one-way ANOVA and Tukey's multiple range test. The variation relative to the mean of each treatment was calculated from the C.V. ( $=SD/\text{mean} \times 100$ ).

The percentage composition of C and N in the tail muscle of *M. moyebi* was lowest in the no-acid, muscle+exoskeleton treatment (mean %C =  $40.4 \pm 0.58\%$ , mean %N =  $10.4 \pm 0.09\%$ ) and highest in the acid-washed, muscle-only and the ground-before-acid treatment (mean %C =  $51.2 \pm 2.28\%$ , mean %N =  $13.6 \pm 0.21\%$ ; Fig. 1). The means for %C and %N of muscle only were significantly higher in the acid-washed and ground-before-acid treatment than in the no-acid treatment (Fig. 1). Removing the exoskeleton did not have a consistent effect between levels of each treatment; thus the mean %C and %N were higher in muscle only than in muscle+exoskeleton for the no-acid treatment but were virtually the same in the acid-washed treatment (Fig. 1). The changes in %C and %N between muscle only and muscle+exoskeleton in the no-acid treatments were similar in magnitude to those found by comparing the effects of

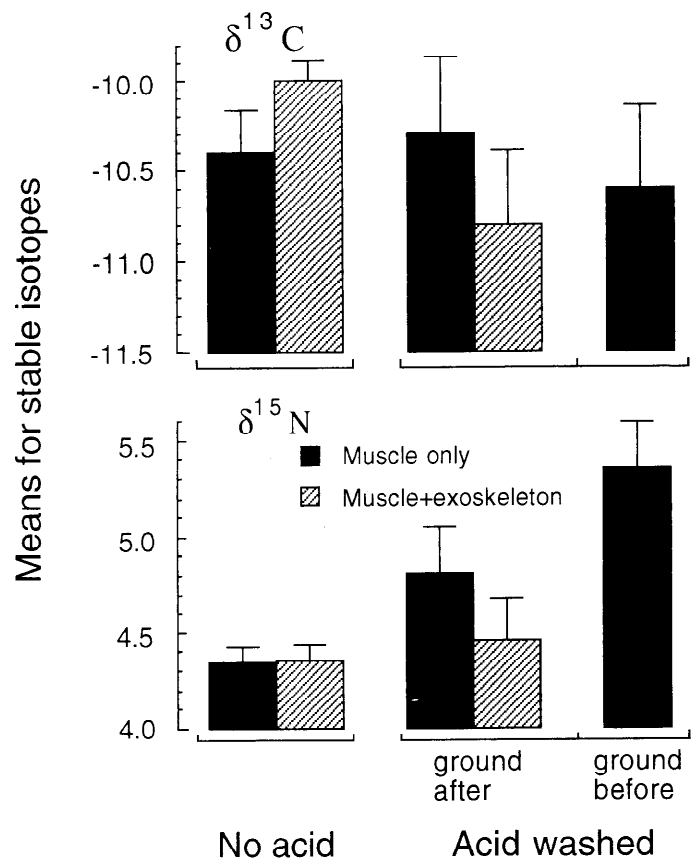


Fig. 2. Mean stable isotope ratios ( $\pm 1$  SE) of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in tail muscle tissue of *Metapenaeus moyebi* for different treatments.

acid washing on shrimp muscle in the muscle+exoskeleton treatments (Fig. 1).

The  $\delta^{13}\text{C}$  values of *M. moyebi* ranged from only  $-10.8\text{‰}$  (acid washed, muscle+exoskeleton) to  $-10.0\text{‰}$  (no acid, muscle only) and did not differ among treatments (Fig. 2). In contrast,  $\delta^{15}\text{N}$  values were significantly higher in the muscle-only, acid-washed treatment (means = 4.8 and 5.3‰) than in the muscle-only, no-acid treatment (mean = 4.3‰; Fig. 2).

The variation in both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  was about two to three times higher in the acid-washed compared with the no-acid treatments. Thus, the C.V. for  $\delta^{13}\text{C}$  ranged from 11.4 to 13% for the acid-washed shrimp compared with 3.4 and 6.7% for the no-acid shrimp (see standard error bars on Fig. 2). For  $\delta^{15}\text{N}$ , the corresponding coefficients of variation were 10–15% for the acid-washed and 5–6% for the no-acid treatments.

Samples of *Enhalus acoroides*, a seagrass with very long (~400 mm), wide leaves (~20 mm), were collected in the Embley River estuary in November 1991. Leaves were thoroughly washed and agitated to remove detritus and loose epiphytes before they were dried to constant weight. Half the leaves in each sample were ground for analysis (no-acid treatment) and the remaining leaves were washed in 1 N HCl for 1 h, rinsed in distilled water (acid

Table 1. Mean composition and stable isotope ratios ( $\pm 1$  SE) of C and N and the results of paired *t*-tests between no-acid and acid-washed leaves for the seagrass *Enhalus acoroides* in the Embley River estuary. *N* = 6 for all comparisons.

Variable	No acid	Acid washed	<i>t</i> -value	<i>P</i>
%C	39.6 $\pm$ 0.37	42.6 $\pm$ 0.50	3.70	0.014
%N	2.8 $\pm$ 0.04	3.2 $\pm$ 0.06	6.01	0.002
$\delta^{13}\text{C}$	-9.6 $\pm$ 0.08	-9.6 $\pm$ 0.07	0.69	0.52
$\delta^{15}\text{N}$	1.58 $\pm$ 0.31	-0.24 $\pm$ 0.36	10.62	<0.001

washed), and then redried to constant weight and analyzed. The proportions of both C and N in *E. acoroides* leaves were higher in acid-washed than no-acid samples, but only by 3% for C and 0.4% for N (Table 1). Although stable carbon isotope values did not differ between acid-washing treatments, the mean  $\delta^{15}\text{N}$  of acid-treated samples was significantly lower than that for the no-acid seagrass (Table 1). Although acid treating had little effect on within-treatment variation for %C, %N, and  $\delta^{13}\text{C}$ , the variation in  $\delta^{15}\text{N}$  values of acid-treated leaves was much higher (C.V. = 369%) than that of the no-acid leaves (C.V. = 48%) (see standard errors on Table 1).

Our results show that there is little justification for acid-rinsing samples of shrimp tail muscle to remove nondietary C in the exoskeleton. Thus, acid washing did not affect the mean  $\delta^{13}\text{C}$  values for juvenile *M. moyebi* and resulted in only a slight change in mean  $\delta^{13}\text{C}$  values for larger *M. bennettiae*. Although the small differences in  $\delta^{13}\text{C}$  recorded for *M. bennettiae* may be significant from a statistical or physiological perspective, these will have little influence on the interpretation of food webs given the far greater variation reported among individuals in space and time (e.g. Bunn and Boon 1993).

In contrast to the mean signatures for  $\delta^{13}\text{C}$ , acid washing enriched the mean  $\delta^{15}\text{N}$  signatures of shrimp tissue to a degree that may confound the interpretation of food webs. This effect of acid washing on  $\delta^{15}\text{N}$  values was greater when shrimp muscle was first ground and then acid washed (Fig. 2). The observed changes in mean  $\delta^{15}\text{N}$  in adult *M. bennettiae* after acid washing ( $\sim 3\%$ ) were of a similar magnitude to those that have been used to infer a change of one trophic level in food-web studies (Fry 1988; Sholto-Douglas et al. 1991). Furthermore, acid-washed samples show an increase in the variation among individuals for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  and therefore a corresponding loss of statistical power (i.e. increasing the risk of a type 2 error).

Goering et al. (1990) also noted changes in  $\delta^{15}\text{N}$  values in some "natural samples" treated with HCl. They suggested that these changes were a result of the different rates of leaching of organic N from compounds with different  $\delta^{15}\text{N}$  values. Goering et al. then selected tissue samples that were free of hard structural components for stable isotope analysis to minimize the possibility of contamination by carbonates in samples that were not acid treated. An alternative strategy would be to dry and grind samples for  $\delta^{15}\text{N}$  analysis, then acid-wash and analyze

samples for  $\delta^{13}\text{C}$ . This would, however, increase the costs of the analyses.

On some CF-IRMS machines, further problems can be encountered with acid-washing plant material that has low levels of N; at low levels of N, we have found that slight variations in the amount of N can lead to marked variations in  $\delta^{15}\text{N}$ , i.e. a source linearity problem (see Fry et al. 1992).

Removing the exoskeleton of larger crustaceans and possibly molluscs, like acid washing, eliminates nondietary carbon; however, removing the exoskeleton does not change the mean  $\delta^{15}\text{N}$  signatures nor increase the among-sample variance. Exoskeleton N is derived from the diet and may be important to include when determining food sources for the consumer. If, however, we are considering crustaceans as a food source for higher order predators, it may not be important to include exoskeleton N because it is unlikely to be assimilated by their predators. Therefore, we may need to obtain estimates of  $\delta^{15}\text{N}$  both with and without the exoskeleton, depending on the objectives of the study.

Our results highlight the importance of testing the effects of sample preparation on stable isotope values. Our findings and those of others (Goering et al. 1990) suggest that acid washing may alter the chemical composition of natural materials, particularly the  $\delta^{15}\text{N}$  values.

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## A comparison of nutrient-limited productivity in *Sargassum natans* from neritic vs. oceanic waters of the western North Atlantic Ocean

**Abstract**—Physiological studies and seawater nutrient analysis showed that the productivity of the macroalga *Sargassum natans* was significantly enhanced by higher N and P availability in neritic compared to oceanic waters of the western North Atlantic Ocean (17–40°N). The initial slope of the  $P$  vs.  $I$  curve ( $\alpha$ ), photosynthetic capacity ( $P_{\max}$ ), dark respiration, and the light saturation irradiance ( $I_s$ ) were all significantly greater in neritic compared to oceanic populations. The higher productivity of neritic *S. natans* correlated with higher levels of tissue N and P; C:N, C:P, and N:P ratios averaged 27.9, 347, and 10.2 in neritic populations compared to 49.4, 877, and 18.1 in oceanic populations. Lower alkaline phosphatase activity in neritic vs. oceanic populations corroborated the higher P limitation in oceanic waters. Experimental pulses with dissolved inorganic N, DIN ( $\text{NO}_3^-$ ), and soluble reactive phosphate, SRP ( $\text{PO}_4^{3-}$ ), significantly enhanced net  $P_{\max}$  and dark respiration of oceanic but not neritic *S. natans*, demonstrating that increased N and P availability enhances productivity of nutrient-depleted *S. natans*. Higher DIN and SRP concentrations within *Sargassum* windrows along shelf fronts in neritic regions explained the higher productivity and suggest that chronic nutrient limitation in oceanic regions is related to highly patchy nutrient supply.

For almost five centuries, the pelagic *Sargassum* community of the North Atlantic Ocean has been a subject of lively debate among explorers and scientists alike. Columbus first described the vegetation, which is comprised

largely of two holopelagic species—*Sargassum natans* and *Sargassum fluitans*—that propagate by vegetative fragmentation. Phycologists have long thought that these plants grow primarily in the Sargasso Sea, where Winge (1923) suggested they grow at “a lively rate.” Parr (1939) was similarly impressed that the entire vegetation seemed vigorous in the central area of the Sargasso Sea. Of the 7–10  $\times 10^6$  t of pelagic *Sargassum* in the Sargasso Sea, ~90% is *S. natans* with the remainder consisting of *S. fluitans* and several species of benthic *Sargassum* recruited from neritic waters of the Caribbean, Gulf of Mexico, Straits of Florida, and the West Indies (Parr 1939).

The precept that pelagic *Sargassum* flourishes in surface waters of the Sargasso Sea is a paradox in biological oceanography (Ryther 1956). Primary productivity in this oligotrophic gyre is characteristically low due to limited vertical nutrient flux to surface waters (Ryther and Menzel 1960). The possible importance of nitrogen fixation by epiphytic cyanobacteria has been considered (Carpenter 1972), although incorporation of fixed nitrogen by *Sargassum* has not been demonstrated. Nitrogen fixation in the oceans can be limited by P, organic matter, and trace metals (Paerl et al. 1987), all of which are of relatively low availability in the nutrient-poor surface waters of the Sargasso Sea. Significant rates of N fixation occur in *Trichodesmium* in the Sargasso Sea and Caribbean (Carpenter and Price 1977) and N fixation by *Trichodes-*