



FEATURE ARTICLE

# Nitrogen stable isotopes in the shell of *Mercenaria mercenaria* trace wastewater inputs from watersheds to estuarine ecosystems

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**ABSTRACT:** We tested the usefulness of  $\delta^{15}\text{N}$  values in the organic matrix of whole shells from *Mercenaria mercenaria* as tracers of anthropogenic nitrogen inputs to coastal ecosystems. Low and high stringency acidification methods were used to define parameters for reliable  $\delta^{15}\text{N}$  determination in shell material for comparison with  $\delta^{15}\text{N}$  values in soft tissues.  $\delta^{15}\text{N}$  values in shell from transplanted and native clams reflected %-wastewater contribution to estuaries, but were 2.3 to 2.5% lighter than  $\delta^{15}\text{N}$  values in soft tissues. Accuracy of  $\delta^{15}\text{N}$  values in shell material depended on recovering a sufficient quantity of organic N from shell (~70  $\mu\text{g}$ ) and was not altered by acidification method. Reliable  $\delta^{15}\text{N}$  values were obtained with as little as 80 mg of shell and using 100  $\mu\text{l}$  of acid, but higher stringency methods (treating more shell with more acid for longer duration) typically yielded more N for subsequent stable isotope analysis. Conversely, higher concentrations of acid reduced N recovery. These results suggest that the content of N recovered was of greater concern to obtaining reliable  $\delta^{15}\text{N}$  values from shell material than acidification effects. Differences between  $\delta^{15}\text{N}$  values in shell material and soft tissues likely reflected differences in N assimilation among tissues. In combination with other analyses, this method may be applied to refine modern and historical trophic assessments and discern natural from anthropogenic influences on coastal ecosystems

**KEY WORDS:** Acidification · Bivalve · Carbonate · Tissue · Nitrogen · Stable isotope · Anthropogenic

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The organic matrix in the shell of *Mercenaria mercenaria* can serve as a record of anthropogenic nitrogen inputs into coastal waters.

Photo: R. H. Carmichael

## INTRODUCTION

Bivalves have long been used as sentinels of ecosystem change. Bivalves are in constant physical and metabolic contact with ocean waters, and accumulate materials or show physiological responses that reflect environmental change (Widdows & Donkin 1991, Chase et al. 2001, Carmichael et al. 2004a,b). Bivalves are also investigated because of their commercial value, plentiful supply, largely sedentary lifestyle, filter feeding habits, and relative tolerance to the action of a variety of agents of change (Farrington et al. 1983,

Price & Pearce 1997). Furthermore, many bivalves live and feed in coastal waters, where they are subject to anthropogenic influences such as wastewater and other chemical inputs associated with urbanization (Burkhardt & Calci 2000, Chase et al. 2001, Valiela 2006). These characteristics make bivalves particularly useful for identifying and tracing sources of anthropogenic N inputs (such as wastewater) to estuaries and defining ecosystem-level effects of eutrophication (Carmichael et al. 2004a,b, Valiela 2006).

Bivalves are potentially powerful recorders of both short and longer-term environmental change. Bivalve soft tissues and shell incorporate organic materials from surrounding waters. While soft tissues are continuously added and turned-over by metabolic processes, shell material is embedded in an aragonite (calcium carbonate-based) matrix, which may be deposited in discrete annual increments and less affected by subsequent metabolic processes (Bayne & Newell 1983, Serban et al. 1988, Rosenberg & Hughes 1991, Risk et al. 1996, Quitmyer & Jones 1997). Hence, soft tissues from bivalves such as *Mytilus edulis*, *Crassostrea virginica*, and other species have been used to monitor relatively short-term variation in trace metals, organic contaminants, and nutrients in marine waters (Chase et al. 2001, Piola et al. 2006, Valiela 2006). Shell material of longer-living bivalves such as *Mercenaria mercenaria* has been used to reconstruct historical ocean bottom temperatures, provide archaeologists with coastal occupation information, and assess historic changes in freshwater flow into aquatic ecosystems (Weidman et al. 1994, Quitmyer & Jones 1997, Schöne et al. 2003). Bivalve soft tissue and shell material, in combination, provide a potentially powerful tool to discern present and past environmental conditions.

Preliminary study suggests it is possible to identify and trace wastewater inputs to estuaries by analyzing the stable isotope ratios of N in bivalve tissue and shell material (Carmichael 2004). Stable isotope ratios are useful for tracing the movement of anthropogenic N through coastal food webs because wastewater-influenced N conveyed by groundwater is isotopically heavier than N derived from atmospheric or fertilizer sources (Peterson & Fry 1987, McClelland et al. 1997). This isotopic signature is detectable, with appropriate fractionation, in nutrients, suspended particulate organic matter, and in tissues of consumers, including bivalves (McClelland et al. 1997, McKinney et al. 2002, Carmichael et al. 2004a, Martinetto et al. 2006). Although there has been little equivalent study on N stable isotope ratios in bivalve shell, preliminary research indicates that shell of transplanted bivalves rapidly acquires  $\delta^{15}\text{N}$  values of wastewater-influenced foods in a host estuary, and shell of modern and fossil *Mercenaria* spp. may be useful to estimate trophic

shifts through time (LeBlanc 1989, O'Donnell et al. 2003, Carmichael 2004). Taken together, these observations suggest that wastewater-derived N may be detectable and traceable in bivalve shell materials in the same way as in soft tissues, providing a unique tool to examine temporal effects of wastewater on coastal systems.

To confidently use  $\delta^{15}\text{N}$  values in bivalve shell to trace N sources or define trophic linkages, it is essential to first define sources of isotopic variability. Although  $\delta^{15}\text{N}$  values in bivalve soft tissues are easily measured, obtaining corresponding ratios in shell material requires special treatment that may affect the stable isotope ratios. Carbonate must be removed before analysis to allow recovery of sufficient organic N, which is present in lower concentrations in shell (0.2 to 0.5%) than in tissues (Avak & Fry 1999, Carmichael 2004). Inorganic carbon removal typically requires relatively stringent acidification. The effects of acidification on elemental ratios in organic N is unclear, but past studies suggest that certain methods alter  $\delta^{15}\text{N}$  values enough to confound interpretation of source inputs and food webs (Table 1). Furthermore, different tissue types of some bivalves show different isotopic fractionation, presumably due to differences in N turnover rates or the physiological energetics of isotopic routing among tissues (Raikow & Hamilton 2001, Lorrain et al. 2002). Because shell material is not metabolized, it also potentially records and retains ontogenic variation in assimilated elements (LeBlanc 1989, O'Donnell et al. 2003). Identifying such sources of variation is particularly important to inform isotopic calibration if N stable isotope ratios in bivalve shells are to be used to assess historical time courses for which soft tissues may not be available.

In this study we tested the usefulness of  $\delta^{15}\text{N}$  values in the organic matrix of whole shells from *Mercenaria mercenaria* as tracers of anthropogenic N inputs to coastal waters. To do this, we determined whether  $\delta^{15}\text{N}$  in shell material reflected %-wastewater contributions to estuaries, and made empirical comparisons between  $\delta^{15}\text{N}$  values in soft tissues and shell material among estuaries receiving different wastewater contributions. To account for potential effects of (1) acidification and (2) variation in N content among shell samples from estuaries receiving different wastewater inputs, we quantified the effects of low and high stringency acidification methods on  $\delta^{15}\text{N}$  values in shell material from native and transplanted clams and identified the quantity of N required to yield reliable  $\delta^{15}\text{N}$  values in shell material. This study is the first to test whether stable isotope techniques may be used to detect differences in anthropogenic N sources recorded in bivalve shells. Our methodology provides a tool for ecologists and others interested in bivalves as sentinels of short and longer-term environmental change.

Table 1. Comparison of acidification (decalcification) methods and effects on  $\delta^{15}\text{N}$  values. Sc = silver capsules, tc = tin capsules, IRMS = isotope ratio mass spectrometry, EA = elemental analyzer

Treatment (acidification)	Incubation time	Analyzer	Sample type	Effect on $\delta^{15}\text{N}$	Source
1% $\text{PtCl}_2$ / 1 N HCl (direct application) 0 to 382 $\mu\text{l}$ , sc 750 to 3500 $\mu\text{l}$ , tc	Until no $\text{CO}_2$ release (4 to 6 d)	PDZ Europa 20-20 IRMS coupled to PDZ Europa EA; GV Instruments Isoprime IRMS coupled to EuroVector EuroEA	<i>Mercenaria mercenaria</i> (clam) shell, tissue	No effect	This study
0.1 N HCl (direct application)	Until no $\text{CO}_2$ release	Finnigan Delta S IRMS	Various arthropods	No effect	Serrano et al. (2008)
1 M HCl (direct application)	3 h	Finnigan MAT-Delta plus IRMS coupled to Thermo/Finnigan FlashEA 1112	Particulate organic matter, sediments, <i>Laminaria hyperborea</i> (seaweed), crustaceans, cephalopods, fish	Variable	Carabel et al. (2006)
1 mol $\text{l}^{-1}$ HCl (direct application)	Until no $\text{CO}_2$ release	Thermo/Finnigan Delta plus IRMS	Various invertebrates	-0.32‰	Jacob et al. (2005)
0.12 N HCl; 20 ml (direct application), tc 12 N HCl (fuming), tc	1 min 4, 8, 12 h	Finnigan Delta S IRMS coupled to Carlo Erba NA 2100 EA	Particulate organic matter	No effect	Lorrain et al. (2003)
1% $\text{PtCl}_2$ / 1 N HCl (direct application, fuming), sc	24 h (fuming)	VG Prism IRMS coupled to Carlo Erba EA	<i>Pleuronectes americanus</i> (fish) tissue <i>Crangon septemspinosa</i> (shrimp) tissue	No effect	Bosley & Wainright (1999)
0.1 N HCl (direct application)	1 h	Europa tracer mass IRMS coupled to Dumass combustion EA	<i>Metapenaeus</i> spp. (shrimp) exoskeleton, tissue <i>Enhalus acoroides</i> (seagrass)	+3.0‰ -1.8‰ (+ variation)	Bunn et al. (1995)
10% EDTA (dialysis) 1 N, 3 N HCl; 20 ml (direct application)	~7 d	VG micromass 602D IRMS after Dumas combustion	<i>Mytilus edulis</i> (mussel) <i>Arctica islandica</i> (clam) Shell only	Variable (no untreated control)	LeBlanc (1989)

## MATERIALS AND METHODS

**Field collection and experimentation. Study sites and native clams:** We collected hard clams, *Mercenaria mercenaria*, from 4 estuaries characterized by a range of watershed urbanization and N loads common to coastal areas (Valiela et al. 1992, Valiela 2006). Study estuaries (and %-wastewater contribution to total N load) were Sage Lot Pond (4%), Green Pond (54%), Childs River (63%) and Snug Harbor (89%) on Cape Cod, MA (Fig. 1). We chose these estuaries because N loading attributes are well studied relative to land-use mosaics on adjacent watersheds (Valiela et al. 1997). Specifically, these estuaries show increased %-wastewater contribution to total N load and corresponding increases in  $\delta^{15}\text{N}$  values in biota relative to increased urbanization over time (Bowen & Valiela 2001, Martinetto et al. 2006). N inputs to these estuaries are primarily conveyed by groundwater, taking

years to move from source to estuary, and varying with large-scale patterns of urbanization and sources of N attenuation (Valiela et al. 1992). Hence, there is little inter-annual or seasonal variation in N stable isotope ratios in primary producers to be conveyed to consumers (McClelland et al. 1997).

Prudently, however, we opted to further normalize for potential anthropogenic or ontogenic variation in stable isotope ratios through time, if any, by selecting young native clams of uniform age (2 to 3 yr), ranging in size from 20 to 60 mm, depending on estuary-specific growth rates (Carmichael et al. 2004b). Harvested clams were held on ice and stored at  $-20^\circ\text{C}$  prior to processing and to aid shucking.

**Transplanted clams:** To determine whether shell of juvenile clams acquired wastewater-specific stable isotope ratios of host estuaries and to define ontogenic differences in N stable isotope ratios between soft tissues and shell, we transplanted 8 to 12 mm hatchery-

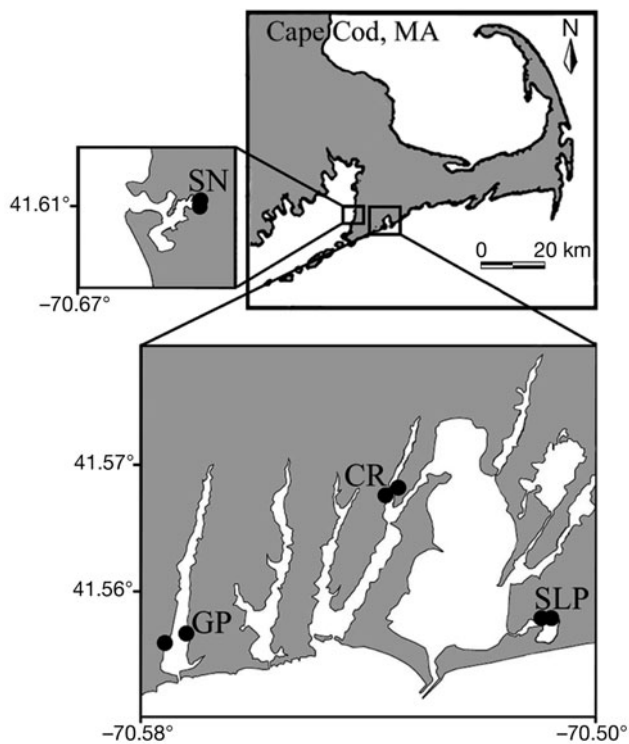


Fig. 1. Study estuaries on Cape Cod, Massachusetts. SLP: Sage Lot Pond, GP: Green Pond, CR: Childs River (all off Nantucket Sound), SN: Snug Harbor (in Buzzards Bay). Bivalve transplant locations (●)

reared clams into each estuary. Juvenile *Mercenaria mercenaria* were obtained from the Aquaculture Research Corporation in Dennis, MA. We opted to use hatchery-reared juveniles because they respond quickly to changes in stable isotope ratio and allow us to compare  $\delta^{15}\text{N}$  values among clams that originated from common seed stock (Carmichael 2004). Clams ( $n = 25$ ) were transplanted into plastic-coated wire mesh aquaculture cages of  $30 \times 30 \times 10$  cm (W  $\times$  L  $\times$  D). Cages were lined on the inside with plastic mesh (6 mm mesh size) and filled with sediment from the estuary into which they were transplanted. Two cages were transplanted at each of 2 locations, for a total of 4 cages per estuary during 2 yr. Clams were removed from each estuary after at least 42 d in 2000 and 84 d in 2001 (final size  $\pm$  SE  $19.4 \pm 1.6$  mm) and stored at  $-20^\circ\text{C}$  prior to processing. The longer transplant time in 2001 was to ensure that transplants reached stable isotopic equilibrium with food sources in host waters.

**Sample preparation. Soft tissue and shell isolation:**

To isolate soft tissues and shells of *Mercenaria mercenaria*, thawed clams were dissected, separating soft tissue from the shell. To prevent contamination from unassimilated food, soft tissues were further dissected to separate the gut from remaining tissues. We opted to use whole soft tissue because preliminary analyses

indicated only a 0.02‰ difference between  $\delta^{15}\text{N}$  in whole soft tissue and adductor muscle of *M. mercenaria*, and to our knowledge whole soft tissue provides the most parsimonious comparison to the organic matrix in whole shell material. Whole soft tissue was dried to a constant weight at  $60^\circ\text{C}$ , ground with a mortar and pestle, and stored in a desiccator.

Whole shells were thoroughly cleaned to remove remnant soft tissues and rinsed with ultrapure  $\text{H}_2\text{O}$ . Whole shells of native clams were coarsely ground in an acid-washed commercial-grade glass blender, followed by fine grinding with a mortar and pestle. For transplanted clams, the 8 to 12 mm of original hatchery-derived shell material was removed, and the remaining shell material was ground with a mortar and pestle. To capture variation but yield a manageable number of samples, samples were processed as aggregates of 10 or more individuals.

**Acidification:** To quantify the effects of acidification methods on  $\delta^{15}\text{N}$  in shell material from native and transplanted clams and identify the quantity of N required to yield reliable  $\delta^{15}\text{N}$  in shell material, we subjected shell and soft tissues (as a proxy for the organic matrix in shell) to low and high stringency acidification methods. Specifically, soft tissues provided a biologically relevant control to test acidification methods, being similar in composition to shell organic matrix but free of carbonate.

**Low stringency:** Different volumes of 1%  $\text{PtCl}_2$  in 1 N HCl were applied serially to samples of shell material (0, 96, 191, 287, 383  $\mu\text{l}$ ) and soft tissues (0, 96, 144, 168, 191, 239  $\mu\text{l}$ ). Acid was directly applied drop-wise to samples pre-measured (3 mg soft tissue; 80 mg shell) into silver capsules (see Table 2). Shell was added to capsules in 20 mg increments, with acid added slowly and allowed to complete bubbling between each addition of acid or shell; 80 mg of shell was chosen based on estimated N content ( $\sim 160$   $\mu\text{g}$ , assuming 0.2% N content of shell) and preliminary acidification trials, which showed that this is the largest quantity of shell that can be serially acidified and concentrated in silver capsules before overflowing or disintegrating the capsules. As negative controls, each acid treatment was applied to empty silver capsules, and  $\sim 20$  mg of unacidified shell (capsule capacity) was packed in silver capsules. As a positive control,  $\sim 3$  mg of unacidified soft tissue was packed in untreated silver capsules. Controls were treated and handled alongside other samples. After final acidification, samples in capsules were dried at  $60^\circ\text{C}$ , closed with forceps, and stored under desiccation before analysis by continuous flow isotope ratio mass spectrometry (IRMS). All samples from transplanted clams were acidified using this low stringency method.

**High stringency:** First, the effects of increasing quantities of relatively low concentrations of acid were



tested by serially applying 1% PtCl<sub>2</sub> in 1 N HCl at 6 quantities (750, 1500, 2000, 2500, 3000, and 3500 µl) to shell material and soft tissues (see Table 2). Acid was pipetted directly onto samples pre-measured (mean ± SE 15.5 ± 0.2 mg soft tissue; 712 ± 4 mg shell) into scintillation vials. The acid was allowed to react with the samples until no further bubbling occurred (4 to 6 d). Second, the effects of increasing acid concentration on N recovery from shell was tested by similarly applying 6 ml of 1% PtCl<sub>2</sub> in HCl at 4 different concentrations (1, 4, 7, and 10 N) to 86 ± 1 mg of shell from native and transplanted clams, which were processed in scintillation vials as described above.

Samples were gently agitated each day during acid incubations. After bubbling ceased, ultrapure water was added to the sample, and the sample was filtered through a pre-combusted 0.7 µm borosilicate glass fiber filter (Whatman GF/F). Vials and filters were thoroughly washed with ultrapure water to minimize residual acid on filters. Filters were dried at 60°C. Shell filters were peeled and packed in tin capsules. Soft tissue was lifted from the filters, ground with a mortar and pestle, and 1 to 3 mg of tissue was packed in tin capsules (subsequent analysis revealed that adhered soft tissues also could be left on filters and packed, without complication). As negative and positive controls, respectively, each acid treatment also was applied to empty scintillation vials and a sample of soft tissues from each site was analyzed un-acidified. These controls were treated and handled alongside other samples. All samples were stored under desiccation before analysis by IRMS.

**Stable isotope analyses.** Samples were analyzed by continuous flow IRMS after sample combustion to N<sub>2</sub> in an online elemental analyzer (EA) at either the University of California Davis Stable Isotope Facility or the Boston University Stable Isotope Laboratory. Samples from native clams were analyzed on a PDZ Europa 20-20 mass spectrometer after combustion in a PDZ Europa Automatic Nitrogen and Carbon Analyzer-Gas Solid Liquid. Gases were separated on a Supelco Carbosieve G column before IRMS. Samples from transplanted clams were combusted in a EuroVector EuroEA elemental analyzer, and combustion gases were separated on a GC column, passed through a reference gas box and introduced into GV Instruments IsoPrime IRMS. Water was removed using a magnesium perchlorate water trap. Each analyzer was calibrated to the expected range of stable isotope ratios for our samples (>10 µg N), accounting for expected low N content of shell organic matter, but remaining within the conventional limits for EA-IRMS. Replicate samples of soft tissues from native clams were passed through both analyzers, as a control, and showed no significant difference in stable isotope values between analyzers. We

also found a mean difference of <0.2‰ between replicate samples passed through each analyzer.

**Statistical analyses.** Regression analysis was used to compare δ<sup>15</sup>N values in soft tissue and shell to wastewater contribution to each estuary and to N content in samples, as well as for comparisons between N recovery and acid concentration. To compare the rate and magnitude of change in stable isotope ratios between shell and soft tissue and between native and transplanted clams, regression analyses were followed by a test for homogeneity of slopes (Sokal & Rohlf 1981) and, when appropriate, analyses of covariance (ANCOVA). Data were log-transformed, as suitable, before testing for significance of regression and higher order statistics. Regression analyses, including *F*-tests, were performed in Microsoft Excel v.11.3.7. ANCOVA and correlation analyses were performed in Stat View 5.0.1. A significance value of *p* < 0.05 was used for all tests.

Each stable isotope ratio data point represents an aggregate of 10 or more individuals, unless otherwise indicated. To capture variation, samples were haphazardly selected for replicate analysis, depending on the number of individuals available from each estuary. Hence, mean δ<sup>15</sup>N values represent data from averages of independently processed, aggregated samples. For example, mean values shown for each estuary (see Fig. 6) represent 19 to 21 aggregates of native clam soft tissues, 4 to 9 aggregates of native clam shells, and 2 to 4 aggregates of transplanted clam soft tissues and shell. Transplant data from different years were averaged because stable isotope ratios did not differ with incubation time, indicating isotopic equilibrium was reached in both years. For Figs. 3 to 5, error bars show the range of variation across 2 to 4 replicate aggregates. All error is reported as standard error. In figures where no error bars are visible, error was smaller than the symbol.

The estimated quantity of N required to obtain reliable δ<sup>15</sup>N values was defined as the quantity above which δ<sup>15</sup>N values reached an asymptote (δ<sup>15</sup>N<sub>max</sub>) when compared to N content (µg) in samples. To make this determination, we calculated the theoretical δ<sup>15</sup>N<sub>max</sub> (horizontal asymptote) for each estuary using a Michaelis-Menten type equation where

$$Y = \frac{\delta^{15}\text{N}_{\text{max}} \times X}{K_m + X} \quad (1)$$

simplified to describe a linear function

$$Y' = \frac{K_m}{\delta^{15}\text{N}_{\text{max}}} X' + \frac{1}{\delta^{15}\text{N}_{\text{max}}} \quad (2)$$

*Y'* and *X'* represent the inverse of data points *y* (δ<sup>15</sup>N values) and *x* (µg N values), (*K<sub>m</sub>*/δ<sup>15</sup>N<sub>max</sub>) is the slope of the line, (1/δ<sup>15</sup>N<sub>max</sub>) is the *y*-intercept, and *K<sub>m</sub>* is the Michaelis constant. We applied this approach to lin-

earize each dataset shown in Fig. 4 and determined  $\delta^{15}\text{N}_{\text{max}}$  from the inverse of the resulting  $y$ -intercept. To normalize the data for variation in magnitude of  $\delta^{15}\text{N}$  values among estuaries, we divided each  $\delta^{15}\text{N}$  ( $y$ -value) by the corresponding theoretical maximum ( $\delta^{15}\text{N}_{\text{max}}$ ) and converted each value to a percentage of  $\delta^{15}\text{N}_{\text{max}}$ . To depict the accuracy of each  $\delta^{15}\text{N}$  value relative to N content of the sample, we combined data among estuaries and plotted % of  $\delta^{15}\text{N}_{\text{max}}$  against N ( $\mu\text{g}$ ).

## RESULTS

### Effects of acidification

$\delta^{15}\text{N}$  values in shell and soft tissues were not significantly altered by acidification methods. After initial acidification, which removed inorganic carbon from shell samples,  $\delta^{15}\text{N}$  values in shell did not change with increasing acid application (Fig. 2A). Soft tissue responded similarly to shell, but had significantly less variation (Fig. 2B), with (mean  $\pm$  SE)  $0.05 \pm 0.01$  and  $0.16 \pm 0.02\delta$  among soft tissue and shell samples, respectively ( $t = 4.18$ ,  $df = 3$ ,  $p = 0.01$ ). All samples showed greater difference in  $\delta^{15}\text{N}$  values among estuaries (reflecting different %-wastewater contributions) than across treatments within estuaries (due to acidifi-

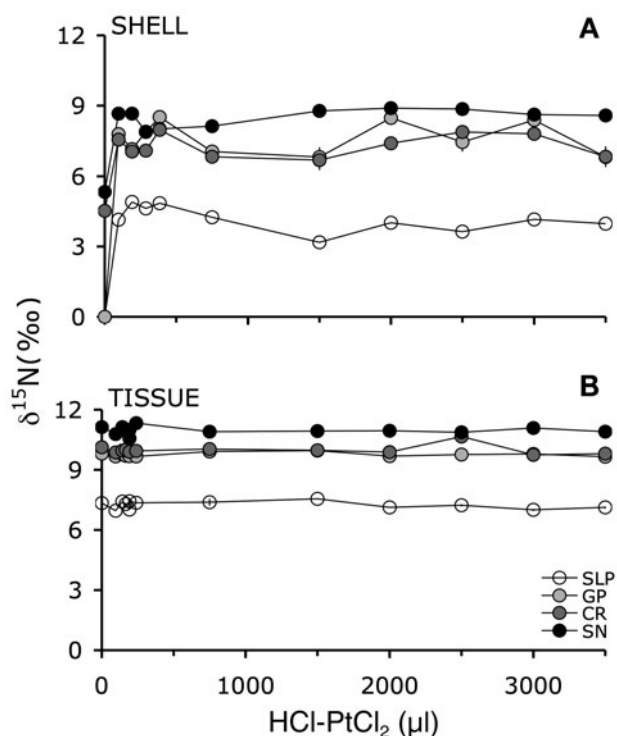


Fig. 2. *Mercenaria mercenaria*.  $\delta^{15}\text{N}$  in (A) shell and (B) soft tissue of native clams from 4 Cape Cod estuaries compared to quantity of acid ( $\text{HCl-PtCl}_2$ ) used for incubation

Table 2. *Mercenaria mercenaria*. Comparison of low and high stringency acidification methods using 1%  $\text{PtCl}_2$  in 1 N  $\text{HCl}$ , including volume of acid applied (treatment), quantity of whole *M. mercenaria* shell treated, and the corresponding quantity and percent (relative to quantity of whole shell treated) of organic N recovered;  $n = 4$  for all treatments. Low and high stringency treatments were 4 and 6 d acid incubations, respectively. Where applicable, values are mean  $\pm$  SE

Treatment ( $\mu\text{g}$ )	Shell (mg)	N ( $\mu\text{g}$ )	% N recovered
<b>Low stringency</b>			
0	20	$11 \pm 1$	0.06
96	80	$64 \pm 4$	0.08
191	80	$73 \pm 7$	0.09
287	80	$63 \pm 5$	0.08
383	80	$63 \pm 3$	0.08
<b>High stringency</b>			
750	$716 \pm 5$	$82 \pm 9$	0.01
1500	$703 \pm 13$	$98 \pm 16$	0.01
2000	$703 \pm 8$	$115 \pm 14$	0.02
2500	$717 \pm 5$	$118 \pm 13$	0.02
3000	$717 \pm 7$	$113 \pm 4$	0.02
3500	$712 \pm 13$	$87 \pm 12$	0.01

cation). To make these comparisons, we combined data from low and high stringency methods (all using 1 N  $\text{HCl}$ ) to yield acid quantity as a continuous variable.

Direct comparison of low and high stringency acidification methods, however, revealed differences in efficiency of each method relative to N recovery (Table 2). Low stringency methods, employing less acid dropwise for up to 4 d, recovered a greater percentage of N than high stringency methods, which used more acid and incubation up to 6 d and included washing with ultrapure water (Table 2). Overall, a greater quantity of N was recovered using the high stringency method because it allowed processing of a larger quantity of shell material. It is important to note that both of these methods used the same, relatively low concentration of acid (1%  $\text{PtCl}_2$  in 1 N  $\text{HCl}$ ), differing only in acid quantity, duration of incubation, and final washing. When we employed high stringency methods, but also increased acid concentration from 1 N to 10 N, recovery of N from shell material decreased significantly (Fig. 3). Responses were similar for native and transplanted clams, hence, data were combined (Fig. 3).

### Nitrogen content

To test whether N recovery may have contributed to variation in  $\delta^{15}\text{N}$  values in shell, we compared  $\delta^{15}\text{N}$  values to N content in each sample.  $\delta^{15}\text{N}$  values in soft tissues and shell showed a significant logarithmic increase as N content increased among samples (Fig. 4,

Table 3), indicating that the accuracy of  $\delta^{15}\text{N}$  decreased when N content of samples was low. Similar responses to low N content have been reported for other sample types (Fry et al. 1992, Avak & Fry 1999). The rate of change in  $\delta^{15}\text{N}$  values with increasing N content was consistent among estuaries (test for homogeneity of slopes, soft tissue:  $F_{3,85} = 1.09$ ,  $p = 0.36$ ; shell:  $F_{3,41} = 1.95$ ,  $p = 0.14$ ), with the magnitude of  $\delta^{15}\text{N}$  values in soft tissues and shell reflecting %-wastewater contribution to each estuary (Fig. 4). Although the initial quantity (weight) of whole shell processed for stable isotope analysis was the same for each estuary, significantly less N was recovered from shells in SLP (ANOVA:  $F_{3,44} = 3.99$ ,  $p = 0.01$ ), the estuary receiving the lowest %-wastewater contribution and the lowest total N load (Fig. 4A).

To quantify the N needed to achieve reliable and less variable  $\delta^{15}\text{N}$  values in soft tissues and shell of

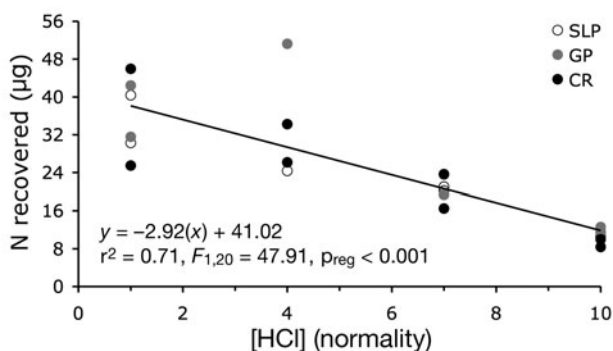


Fig. 3. *Mercenaria mercenaria*. Quantity of N recovered from shell samples (collected from 3 Cape Cod estuaries, for definitions see Fig. 1) incubated with increasing concentrations of acid. All samples were treated with 1%  $\text{PtCl}_2$  in HCl. Since there was no difference in N recovery between age classes (test for homogeneity of slopes:  $F_{1,18} = 3.34$ ,  $p = 0.08$ ), data from native clams and transplanted juveniles were combined

Table 3. Regression statistics for  $\delta^{15}\text{N}$  (‰) in shell and soft tissue of native clams compared to N ( $\mu\text{g}$ ) per sample (shown in Fig. 4). For site definitions, see Fig. 1

	$F_{\text{reg}}$	p	df	$r^2$	y
<b>Shell</b>					
SLP	40.38	<0.001	1,8	0.83	$1.82 \ln(x) - 3.81$
GP	66.14	<0.001	1,11	0.86	$3.09 \ln(x) - 5.43$
CR	46.01	<0.001	1,13	0.78	$1.30 \ln(x) + 1.69$
SN	89.90	<0.001	1,9	0.91	$1.53 \ln(x) + 1.50$
<b>Tissue</b>					
SLP	97.87	<0.001	1,22	0.82	$1.60 \ln(x) - 1.68$
GP	182.46	<0.001	1,20	0.90	$1.42 \ln(x) + 2.37$
CR	184.24	<0.001	1,21	0.90	$1.66 \ln(x) + 1.03$
SN	266.13	<0.001	1,22	0.92	$1.66 \ln(x) + 1.95$

*Mercenaria mercenaria*, we calculated the asymptotic  $\delta^{15}\text{N}$  value ( $\delta^{15}\text{N}_{\text{max}}$ ) approached by our data relative to estuary-specific wastewater contributions (Table 4). Although we applied a logarithmic model to test the significance of our data in Fig. 4, we recognize that there is a maximum quantity of N measurable by standard IRMS, and within that limit our data approached an average asymptotic  $\delta^{15}\text{N}$  value (Fig. 4) that should represent an accurate maximum  $\delta^{15}\text{N}$  value in *M. mercenaria* soft tissue or shell from each estuary when sufficient N is analyzed from a sample (Table 4,  $\delta^{15}\text{N}_{\text{max}}$ ). We then determined the relative accuracy of each measured data point by calculating the % of  $\delta^{15}\text{N}_{\text{max}}$  achieved by each sample (Fig. 5). Since the linear regression used to determine  $\delta^{15}\text{N}_{\text{max}}$  for SLP was not significant for shell and had a significantly different slope from other estuaries for soft tissues (Table 4; test for homogeneity of slopes,  $F_{3,84} = 31.35$ ,  $p < 0.001$ ), we excluded SLP data from further analysis. Since the slopes of the remaining regression lines did not vary among estuaries (Table 4; test for homogeneity of slopes, tissue:  $F_{2,63} = 2.43$ ,  $p = 0.10$ ; shell:  $F_{2,37} = 0.03$ ,  $p = 0.99$ ), and calculating % of  $\delta^{15}\text{N}_{\text{max}}$  normalized our data relative to estuary-specific differences in  $\delta^{15}\text{N}$  values, we combined datasets among the remaining estuaries (keeping soft tissue and shell data separate). This approach improved the power of our comparisons and yielded a single equation for the relationship between % of  $\delta^{15}\text{N}_{\text{max}}$  and N ( $\mu\text{g}$ ) for shell (Fig. 5) and soft

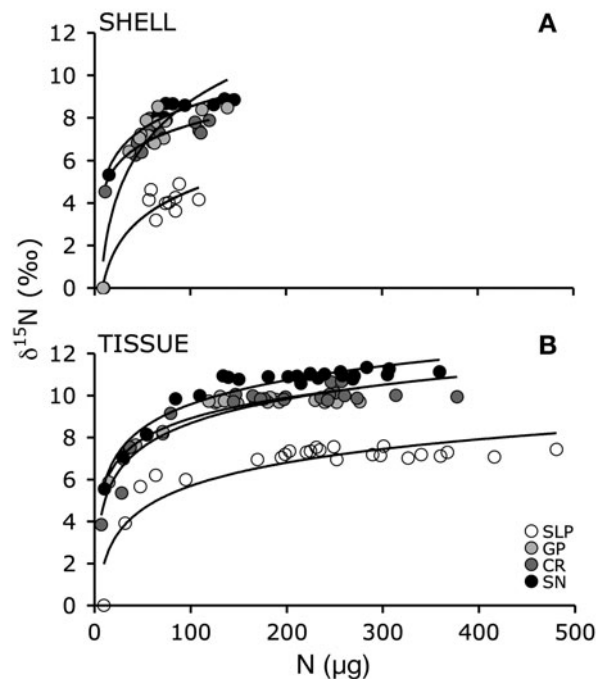


Fig. 4. *Mercenaria mercenaria*.  $\delta^{15}\text{N}$  in (A) shell and (B) soft tissue of native clams compared to N ( $\mu\text{g}$ ) per sample. Regression statistics are shown in Table 3, site definitions in Fig. 1

Table 4. Regression statistics for the linearized Michaelis-Menten type equation used to determine  $\delta^{15}\text{N}_{\text{max}}$  (horizontal asymptote) for shell and soft tissue data from each estuary (shown in Fig. 4), compared to mean measured  $\delta^{15}\text{N}$  values and the resulting % of  $\delta^{15}\text{N}_{\text{max}}$  achieved by the measured values. Measured  $\delta^{15}\text{N}$  values correspond to native clam data shown in Fig. 6, and include only samples with  $\geq 70 \mu\text{g N}$ . Where applicable, values are mean  $\pm$  SE

	$F_{\text{reg}}$	p	df	$r^2$	y	$\delta^{15}\text{N}_{\text{max}}$	Measured $\delta^{15}\text{N}$	% of $\delta^{15}\text{N}_{\text{max}}$
<b>Shell</b>								
SLP	0.15	0.71	1,7	0.02	$1.65x + 0.23$	$4.44 \pm 0.97$	$4.16 \pm 0.17$	$94 \pm 4$
GP	14.07	<0.01	1,10	0.58	$1.72x + 0.11$	$9.49 \pm 0.71$	$7.94 \pm 0.33$	$84 \pm 3$
CR	11.96	<0.01	1,12	0.50	$1.48x + 0.12$	$8.56 \pm 0.51$	$7.53 \pm 0.13$	$88 \pm 2$
SN	11.53	<0.01	1,8	0.59	$1.24x + 0.10$	$9.59 \pm 0.38$	$8.55 \pm 0.12$	$89 \pm 1$
<b>Tissue</b>								
SLP	160.93	<0.001	1,21	0.88	$3.51x + 0.12$	$8.06 \pm 0.17$	$7.18 \pm 0.08$	$89 \pm 1$
GP	352.99	<0.001	1,20	0.95	$1.20x + 0.10$	$10.32 \pm 0.13$	$9.44 \pm 0.18$	$92 \pm 2$
CR	239.19	<0.001	1,21	0.92	$1.24x + 0.10$	$10.37 \pm 0.27$	$9.96 \pm 0.07$	$96 \pm 1$
SN	274.27	<0.001	1,22	0.93	$1.03x + 0.09$	$11.35 \pm 0.32$	$10.86 \pm 0.08$	$96 \pm 1$

tissue (data not shown, but regression statistics are provided in the legend to Fig. 5). The resulting % of  $\delta^{15}\text{N}_{\text{max}}$  achieved by each sample increased significantly as N content increased, with the majority of samples reaching 80 % of  $\delta^{15}\text{N}_{\text{max}}$  when N content was 70 to 80  $\mu\text{g}$  (Fig. 5).

Eliminating all data points for which N content was <70  $\mu\text{g}$  yielded average  $\delta^{15}\text{N}$  values for native *Mercenaria mercenaria* from each estuary that fell within 84 to 94 % of  $\delta^{15}\text{N}_{\text{max}}$  for shell and within 89 to 96 % of  $\delta^{15}\text{N}_{\text{max}}$  for soft tissues (Table 4, measured  $\delta^{15}\text{N}$ ). Juvenile transplants, from which only newly deposited shell was used, were too small to yield any samples with >70  $\mu\text{g}$  N in tissue or shell. Despite this low N content,  $\delta^{15}\text{N}$  values fell at  $84 \pm 2$  % of  $\delta^{15}\text{N}_{\text{max}}$ , close to higher N samples (data not shown).

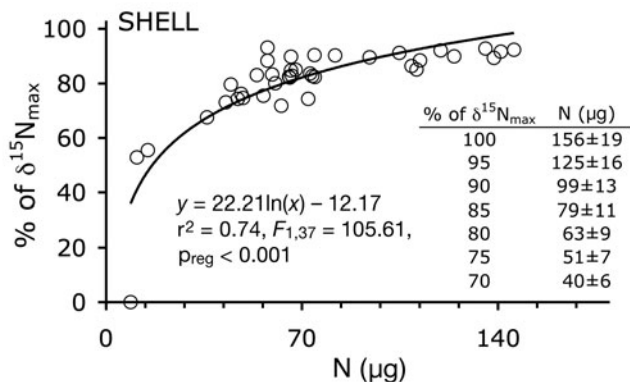


Fig. 5. *Mercenaria mercenaria*. Percent of  $\delta^{15}\text{N}_{\text{max}}$  achieved by each shell sample compared to N content ( $\mu\text{g}$ ) in the sample, for all estuaries combined. Inset shows the quantity of N (mean  $\pm$  SE) required to achieve measured  $\delta^{15}\text{N}$  values in shell within 70 to 100 % of  $\delta^{15}\text{N}_{\text{max}}$ , calculated from the equation for the best fit line shown. A similar relationship was derived for soft tissue (data not shown:  $y = 14.80 \ln(x) - 16.70$ ,  $R^2 = 0.90$ ,  $F_{1,67} = 97.99$ ,  $p_{\text{reg}} < 0.001$ )

### Tracing wastewater inputs

To determine whether  $\delta^{15}\text{N}$  values in *Mercenaria mercenaria* shell reflected differences in anthropogenic N sources among estuaries, we compared  $\delta^{15}\text{N}$  values in soft tissues and shell with %-wastewater contribution to total nitrogen load to each estuary. To make these comparisons we used only data points for which N content was >70  $\mu\text{g}$  N for native clams and all data points ( $\leq 20 \mu\text{g}$  N) for transplanted juveniles.  $\delta^{15}\text{N}$  values in soft tissues and shell from native clams increased significantly with

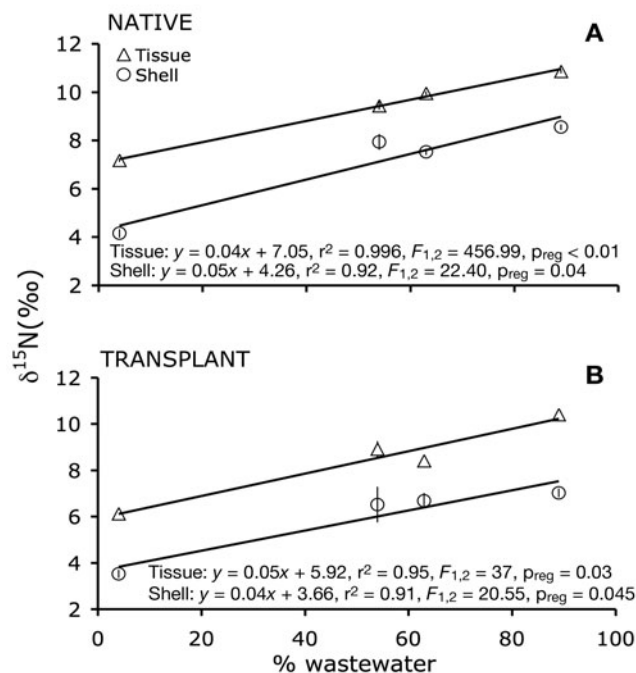


Fig. 6. *Mercenaria mercenaria*. Mean  $\delta^{15}\text{N}$  in shell and soft tissue compared to % contribution of wastewater to Cape Cod estuaries for (A) native clams and (B) transplanted juveniles



increasing %-wastewater contribution to receiving estuaries (Fig. 6).  $\delta^{15}\text{N}$  in juvenile transplants reflected shifts in anthropogenic N sources similar to native clams (Fig. 6), despite possible depletion of  $\delta^{15}\text{N}$  values due to low N content (transplanted juvenile samples represent only newly deposited shell material and  $\leq 20 \mu\text{g N}$ ). Although soft tissues and shell responded similarly to changes in wastewater contribution in each case (test for homogeneity of slopes, native:  $F_{1,4} = 0.66$ ,  $p = 0.47$ ; transplants:  $F_{1,4} = 0.16$ ,  $p = 0.71$ ),  $\delta^{15}\text{N}$  values in shell were consistently lighter than in soft tissues, showing a mean difference of  $2.3 \pm 0.3\%$  among natives (ANOVA:  $F_{1,5} = 46.72$ ,  $p = 0.001$ ) and  $2.5 \pm 0.3\%$  among transplanted juveniles (ANOVA:  $F_{1,4} = 52.05$ ,  $p < 0.001$ ).

Transplanted juveniles also had significantly lighter  $\delta^{15}\text{N}$  values than native clams (Fig. 6; paired  $t$ -test, tissue:  $t_3 = 3.53$ ,  $p = 0.02$ ; shell:  $t_3 = 5.00$ ,  $p = 0.01$ ).  $\delta^{15}\text{N}$  values in soft tissues ranged from 7.2 to 10.9‰ for natives versus 6.1 to 10.4‰ for transplants and in shell ranged from 4.2 to 8.6‰ for natives versus 3.5 to 7.0‰ for transplants, resulting in a mean difference of  $0.9 \pm 3\%$  for tissues and  $1.1 \pm 0.2\%$  for shell between natives and transplants.

## DISCUSSION

Since N stable isotope ratios have not been extensively measured in bivalve shells and sample preparation methods have not been rigorously tested (Table 1), we sought to determine whether sample processing methods may have a significant effect on reliable determination of  $\delta^{15}\text{N}$  values in highly carbonaceous bivalve shell. To define and eliminate sources of variation in our analyses, we tested methodological robustness by accounting for potential effects of acidification as well as variation in N content among samples. This approach was the most parsimonious and readily measurable alternative, but also provided an opportunity to help resolve differences among previous studies and more rigorously test methods that have not been thoroughly assessed for any sample type (Table 1). Furthermore, calibration of  $\delta^{15}\text{N}$  values in bivalve shell relative to wastewater influences is needed to confidently use these data to trace N sources or define trophic linkages through time or among locations. These data will be extremely useful to reconstruct historical anthropogenic nutrient inputs or trophic communities particularly when soft tissues are not available.

### Acidification and nitrogen content

Although acidification methods did not directly alter  $\delta^{15}\text{N}$  values, our results indicate a relationship between acidification methods and N recovery that indi-

rectly affected  $\delta^{15}\text{N}$  values. Acidification with higher concentrations of acid or using low concentrations followed by washing (Fig. 3, Table 2) likely resulted in lower N recovery due to disassociating shell matrix protein complexes and solubilizing some protein (LeBlanc 1989, Salt et al. 1982, Wilbur & Saleuddin 1983, Serrano et al. 2008). The lighter  $\delta^{15}\text{N}$  values in shell and soft tissue that are low in N content (Fig. 4) illustrates a mechanism that explains variation in  $\delta^{15}\text{N}$  values due to acidification methods, which has not been explicitly recognized or quantified in previous studies (Table 1; LeBlanc 1989, Jacob et al. 2005, Carabel et al. 2006). The Michaelis-Menten based analysis we tested (Fig. 5) proved a statistically robust method to quantify the minimum amount of N required to obtain reliable  $\delta^{15}\text{N}$  values in both soft tissues and shell that could be applied to other tissue types. Comparison of these results with the regressions in Fig. 4, however, suggests that regression analyses of isotope values against N content may be sufficient to roughly estimate minimum N content and guide analyses when precise values of  $\delta^{15}\text{N}_{\text{max}}$  are not needed. These findings suggest that N quantity, rather than a change due to acidification, was responsible for variation in and artificial lightening of  $\delta^{15}\text{N}$  values (Fig. 4, Table 1).

This mechanism (lower N content = less reliable  $\delta^{15}\text{N}$  values) explains much of the observed variation in  $\delta^{15}\text{N}$  values throughout this study and is consistent with previous reports. (1) Greater variation in  $\delta^{15}\text{N}$  values observed in shell compared to soft tissues (Fig. 2) is consistent with variation in N removal efficiency (Table 2); past methodological and other analyses of stable isotope ratios have rarely reported N removal efficiency and/or N content of samples (Table 1). (2) Although  $\delta^{15}\text{N}$  values in transplanted clams reflected N source shifts among estuaries similar to native clams (Fig. 6), all of these samples yielded  $\leq 20 \mu\text{g N}$  and showed a consistently lower magnitude ( $\sim 1\%$  depletion) in  $\delta^{15}\text{N}$  values; this result is consistent with a previous finding that samples containing  $< 20 \mu\text{g N}$  deviated as much as 0.6‰ from expected values (Avak & Fry 1999). (3) We found lower N content and correspondingly greater variation in  $\delta^{15}\text{N}$  values in clam shells from SLP, the least N-loaded estuary (Fig. 4), suggesting that spatial variation in N quantity as well as source changes may affect  $\delta^{15}\text{N}$  values. Other studies have shown similar relationships between N inputs and N content in resident biota (Kinney & Roman 1998, Hauxwell et al. 2003, Carmichael et al. 2004b), but have not examined relationships between N content and  $\delta^{15}\text{N}$  values. These findings indicate that analysis of  $\delta^{15}\text{N}$  in shell may be complicated by the ability to isolate and concentrate a minimum quantity of N, which may in turn depend on processing methods, N inputs to the sampling site, or other factors that affect N content in samples.

Conventional methods for stable isotope analysis include coupling an elemental analyzer to a stable isotope ratio mass spectrometer (EA-IRMS system, Table 1), which can be calibrated to manage low N samples ( $>10 \mu\text{g N}$ ). Our findings indicate that even with low N calibration, when using conventional EA-IRMS, samples containing  $<70 \mu\text{g N}$  had higher variation and significantly lighter  $\delta^{15}\text{N}$  values compared to higher N content samples. Recent study shows that the conventional EA-IRMS system can be modified to accommodate samples containing  $>1$  to  $2 \mu\text{g N}$ , but as these samples require substantial care to avoid contamination, this method is not widely used or accessible, and has not been attempted with high carbonate samples such as bivalve shell (Carman & Fry 2002). It is important to know N content of different sample types from different locations prior to stable isotope analysis and select the appropriate method for analysis. If sufficient N is obtained, however, our results suggest that these samples can be acidified according to conventional methods without concern for altering  $\delta^{15}\text{N}$  values.

### Tracing wastewater inputs

Using these methods, we found that shell material from *Mercenaria mercenaria*, like soft tissues, reliably acquired  $\delta^{15}\text{N}$  values that reflected estuary-specific anthropogenic N sources (Fig. 6), but with a roughly 2.4‰ depletion. Hence, these data suggest a correction factor of 2.4‰ is needed to use  $\delta^{15}\text{N}$  values in shell material alone for trophic assessments in this species. Unlike other variation in our data, the tissue-specific differences in  $\delta^{15}\text{N}$  values cannot be explained by differences in N content, since both shell and soft tissues yielded sufficient N for reliable analysis (Figs. 2 & 6). Thus, this difference is not likely related to sample processing methods. Since shell material is not metabolized like soft tissues, shell also has potential to record changes in anthropogenic N sources through time or capture ontogenic variation in N processing. It is, therefore, possible that using whole native shells for some analyses rather than only the ventral margin (most recently deposited shell material), resulted in an aggregate  $\delta^{15}\text{N}$  value in shell organic matrix that was lighter compared to soft tissues in native clams.

### Isotopic differences between shell and soft tissue

Close examination of our data, however, suggested that neither anthropogenic nor ontogenic factors were responsible for the difference in  $\delta^{15}\text{N}$  values between

soft tissues and whole shell of native clams in this study. (1)  $\delta^{15}\text{N}$  values in juvenile transplants, for which we only analyzed shell deposited during the study, showed the same difference in  $\delta^{15}\text{N}$  values between shell and tissue; if differences in  $\delta^{15}\text{N}$  values between tissue and shell were due to anthropogenic source shifts or ontogeny, we would expect transplanted juveniles to show no difference in these  $\delta^{15}\text{N}$  values. (2) The difference in  $\delta^{15}\text{N}$  between tissue and shell was consistent among estuaries, regardless of increasing%-wastewater contribution (Fig. 6); if there were a measurable shift in anthropogenic N sources in any host estuary during the short time (2 to 3 yr) that young native clams were growing, we would expect  $\delta^{15}\text{N}$  values in tissue and shell to diverge with increasing wastewater input rather than remain constant across all estuaries. (3) The potential contribution of ontogeny to variation in  $\delta^{15}\text{N}$  values between native and transplanted clams is likely small, if present; native clams were young, of uniform age (2 to 3 yr), and the small ( $\sim 1\%$ ) difference in  $\delta^{15}\text{N}$  values between native and transplanted clams corresponded with expected low N recovery from transplanted clams (Avak & Fry 1999, this study).

Since our analyses strongly suggest that the difference in  $\delta^{15}\text{N}$  values between soft tissue and shell was not a methodological artifact, a physiological explanation seems more likely. Shell formation is a complex process, coupling physiochemical processes that build  $\text{CaCO}_3$  crystals with cellular processes that intercollate the organic matrix. Shell formation, therefore, can account for a high proportion of the total energy required for growth, particularly among thickly shelled bivalves such as *Mercenaria mercenaria* (Wilbur & Saleuddin 1983, Rosenberg & Hughes 1991). Bivalves could benefit energetically by assimilating lighter forms of N or lighter amino acids into shell, resulting in relatively depleted organic matrix in shell compared to soft tissues (Rosenberg & Hughes 1991). For example, Carmichael (2004) and Carmichael et al. (2004c) found significantly heavier  $\delta^{15}\text{N}$  values in *M. mercenaria* soft tissues compared to other bivalves. Lighter  $\delta^{15}\text{N}$  values in shell are, therefore, consistent with possible mass balance of N stable isotope ratios in this species. Accordingly, LeBlanc (1989) reported a small mean difference ( $-0.1 \pm 0.2\%$ ) between soft tissue and shell  $\delta^{15}\text{N}$  values in the mussel *Mytilus edulis*, which has a much thinner shell. In the same study, a thicker shelled bivalve, *A. islandica*, showed a potential soft tissue to shell offset similar to *M. mercenaria* in this study ( $\delta^{15}\text{N}_{\text{tissue}} - \delta^{15}\text{N}_{\text{shell}} = 2.7\%$ ; LeBlanc 1989). Additional studies are needed to resolve possible physiological mechanisms for this interspecies variation in  $\delta^{15}\text{N}$  values between shell and soft tissue.

### Comparison to other studies

O'Donnell et al. (2003) reported somewhat heavier  $\delta^{15}\text{N}$  values as well as a smaller difference between  $\delta^{15}\text{N}$  values in soft tissue and shell in *Mercenaria mercenaria* from the southeastern USA ( $\delta^{15}\text{N}_{\text{tissue}} - \delta^{15}\text{N}_{\text{shell}} = 0.7\text{‰}$ ;) compared to this study. Regional and temporal differences in available foods, N source inputs, as well as environmental attributes may affect shell composition, resource allocation, or physiological processing of foods in ways that alter stable isotope ratios (Krantz et al. 1987, Raikow & Hamilton 2001, Goodfriend & Weidman 2001, Lorrain et al. 2002, O'Donnell et al. 2003). In this study, we also analyzed whole tissue for comparison to whole shell and processed both sample types in the same manner, while O'Donnell et al. (2003) averaged  $\delta^{15}\text{N}$  values from separately analyzed foot, mantle, and muscle tissue. Soft tissue samples also were treated differently from one another and differently from shell material in the O'Donnell et al. (2003) study. These differences in sample processing and analysis may contribute to some difference in reported  $\delta^{15}\text{N}$  values between studies. We cannot fully evaluate the significance of these differences because O'Donnell et al. (2003) did not explicitly report the method of shell sample acidification or N content of analyzed samples. These comparisons, however, re-emphasize the need to understand various sources of spatial and temporal variation as well as differences in sample processing among studies.

Our data have important implications for using  $\delta^{15}\text{N}$  values in shell to historically trace changes in anthropogenic N sources or assess trophic relationships, whether making comparisons among long-lived individuals or comparing modern to ancient samples. O'Donnell et al. (2003) compared  $\delta^{15}\text{N}$  values between modern and fossil *Mercenaria mercenaria* shells and attributed differences to a shift in diet composition through time. Since many bivalves are selective and all are primary consumers, significant changes in  $\delta^{15}\text{N}$  values seem unlikely to reflect a substantial change in trophic position (Carmichael et al. 2004a–d). Shifts in anthropogenic N sources, however, have certainly occurred across locations and in the eras between the lifetimes of ancient and modern samples. If ignored, N source shifts and the consistent difference between shell and soft tissues (0.7 to 2.4‰, depending on processing method) are sufficient to mislead historical assessments of trophic position based on  $\delta^{15}\text{N}$  values in shell. Our data point out uncertainty in assessments that attribute shifts in  $\delta^{15}\text{N}$  values through time and space to changes in diet composition without consideration of N source changes or other variables.

### CONCLUSIONS

Percent wastewater contribution to receiving estuaries and N content analyzed were the most important factors affecting  $\delta^{15}\text{N}$  values in soft tissues and shell of *Mercenaria mercenaria* in this study. Our data highlight the importance of considering how anthropogenic N sources may have changed through time, when applying  $\delta^{15}\text{N}$  values in shell for ecological assessments. Furthermore, attention to N content in samples and use of simple established correction factors may reduce variation in, and increase accuracy of,  $\delta^{15}\text{N}$  values in shell and other highly inorganic or low N content samples. The quantity of N recovered from each sample depended on the initial quantity of material processed and concentration of acid, as well as the initial N content of the sample (which may have varied with the anthropogenic N input to the water body from which the sample was collected). Based on our efforts, therefore, we suggest that a suitable approach to limit potential sources of variation in  $\delta^{15}\text{N}$  values in shell is to analyze as much material as possible using relatively low acid concentrations (e.g. 200 mg of shell, treated with 2 ml of 1%  $\text{PtCl}_2$  in 1 N HCl for 2 d). Other factors such as environmental variation, ontogeny, and physiology did not appear to significantly affect  $\delta^{15}\text{N}$  values in samples across sites, but may have accounted for regional differences in  $\delta^{15}\text{N}$  values between this study and others. Our data suggest, however, that physiological differences in N assimilation into shell may explain the significant depletion of  $\delta^{15}\text{N}$  values in shell compared to soft tissues.

Overall, we provide evidence that stable isotope techniques can detect shifts in anthropogenic N sources recorded in bivalve shells, and we make our methodology readily accessible for ecologists and others interested in bivalves as sentinels of short and longer-term environmental change. As stable isotope analysis becomes an increasingly useful and utilized tool for a variety of ecological studies, there is increasing responsibility to describe processing methods and controls as explicitly and clearly as possible to allow comparison among studies, across locations, and through time. In particular, shell and tissue preparation methods must be rigorously tested and calibrated among species and locations. Our methodological analyses provide a first step to inform future isotope analyses by defining method-specific effects on stable isotope ratios that were not previously thoroughly evaluated.  $\delta^{15}\text{N}$  in shell, in combination with sclerochronological aging techniques and other elemental analyses, can refine modern and historical diet analyses, trace N entry to coastal waters relative to changes in land use on adjacent watersheds, define relationships between nutrient inputs, climate

changes and bivalve growth and survival, and help discern natural from anthropogenic influences on coastal ecosystems.

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