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Temporal and spatial variability in stable isotope compositions of a freshwater mussel: implications for biomonitoring and ecological studies

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Abstract Stable isotopes can be used to elucidate ecological relationships in community and trophic studies. Findings are calibrated against baselines, e.g. from a producer or primary consumer, assumed to act as a reference to the isotopic context created by spatio-temporal attributes such as geography, climate, nutrient, and energy sources. The ability of an organism to accurately represent a community base depends on how, and over what time-scale, it assimilates ambient materials. Freshwater mussels have served as references for trophic studies of freshwater communities and as indicators of change in nutrient pollution load or source. Their suitability as reference animals has not yet been fully explored, however. We conducted a series of studies examining the suitability of freshwater mussels as isotopic baselines, using their ability to reflect variation in ambient nutrient loads as a case scenario. (1) We analyzed bivalve foot tissue $\delta^{15}N$ and $\delta^{13}C$ from 22 stream reaches in the Piedmont region of North Carolina, USA to show that compositions varied substantially among locations. Site mean bivalve $\delta^{13}C$ values correlated with site ambient particulate organic matter (POM) $\delta^{13}C$ values, and site mean bivalve $\delta^{15}N$ values correlated with site ambient water dissolved $\delta^{15}N\text{-}NO_3$ values. (2) Similarity of results among sample types demonstrated that the minimally invasive hemolymph sample is a suitable substitute for foot tissue in $\delta^{15}N$ analyses, and that small sample sizes generate means representative of a larger population. Both findings can help minimize the impact of sampling on imperiled freshwater mussel populations. (3) In a bivalve transplantation study we showed that hemolymph $\delta^{15}N$ compositions responded to a shift in ambient dissolved $\delta^{15}N\text{-}NO_3$, although slowly. The tissue turnover time for bivalve hemolymph was 113 days. We conclude that bivalves serve best as biomonitors of chronic, rather than acute, fluctuations in stream nutrient loads, and provide initial evidence of their suitability

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as time-integrated isotopic baselines for community studies.

Keywords *Elliptio complanata* · $\delta^{15}N$ · $\delta^{13}C$ · Nutrient loading · Tissue turnover · Isotopic baseline

Introduction

Stable isotope studies have generated inferences about trophic relationships (O'Reilly et al. 2002), nutritional conditions (Gannes et al. 1997, 1998), and even the migratory origins of community members (Hobson 1999; Rubenstein and Hobson 2004). Processes that cycle nutrients within and between biological compartments affect the isotopic composition of recipients (Criss 1999; Rubenstein and Hobson 2004). Isotopic compositions of animals and plants are, however, also affected by the isotopic composition of the nutrients and organic compounds forming the base of their food web. Signatures reflect, for example, the extent and source of nutrient contributions, e.g. allochthonous or autochthonous, or littoral or pelagic, pathways (France 1995; Post 2002), marine or freshwater origins (Bilby et al. 1996; Helfield and Naiman 2001), or via different sources of nutrient pollution (McClelland and Valiela 1998; Showers et al. 1990, 1999). Consequently, isotopic compositions of biological communities can vary over space and over time (Cabana and Rasmussen 1996; Post 2002; Rubenstein and Hobson 2004).

Isotopic surveys typically account for this potential variation by standardizing results against "baseline" signatures of organisms at or near the base of the food web. The accuracy, and appropriate choice, of a baseline representative for calibration of isotopic surveys is critical to the quality of inferences about higher-order functions or multiple-system comparisons (Post et al. 2000). When baseline estimates poorly represent the food web base, or represent only one point in a continuum that varies over time (e.g. with seasonal or stochastic changes in nutrient source and quantity), ecological-level predictions can falter (O'Reilly et al. 2002; Post et al. 2000). Reviews repeatedly call for more careful characterization of isotopic baselines in ecological studies (Gannes et al. 1997; Lorraine et al. 2002; O'Reilly et al. 2002; Post 2002; Rubenstein and Hobson 2004).

Bivalves have received attention for their potential role as time-averaged integrators of the more ephemeral isotopic signatures of short-lived producer organisms (Cabana and Rasmussen 1996; Post 2002). Freshwater bivalves, as filter-feeders, remove and assimilate algae, detritus, and microorganisms from the water column. Predominantly sessile, relatively long-lived, and faced as a faunal group with precipitous declines in recent decades, they have been regarded as sensitive indicators of riverine habitat character and change. Their longevity, limited movement, relative ease of collection, and low trophic position, also make them suitable candidates for estimation of baseline isotopic signatures. Similarly to the way that inorganic isotopes recorded in bivalve shells are used to reconstruct past climatic conditions (Jones and Quitmyer 1998), the isotopic composition of organic compounds (e.g. nitrogen, carbon, and sulfur) in bivalve tissues, hemolymph, or shells may reflect, and potentially chronicle, shifts in habitat-use or land-use and the source of associated nutrient loads (McKinney et al. 1999; Raikow and Hamilton 2001; Post 2002).

One very important driver of variation in both the health and isotopic composition of freshwater communities is nutrient pollution. Nutrient pollution, the anthropogenic influx of nitrogen and phosphorus compounds (e.g. from agricultural and residential run-off; Allan 1995), is a serious threat to freshwater and coastal ecosystems. Excessive accumulation of nutrients in fresh waters and estuaries can cause eutrophication, periodic harmful algal blooms, bottom anoxia, associated fish kills, and, ultimately, shifts in ecosystem dynamics and the abundance and diversity of resident biota (Nixon 1995; Paerl 1988, 1997). The problem has reached critical levels in recent years, with varied consequences to commercial fishing, public health, and recreation (Knowler 2000). Stable isotope techniques have greatly advanced efforts to identify non-point sources of surface water nutrient pollution (McClelland and Valiela 1998; Showers et al. 1990, 1999). Stable nitrogen and oxygen isotopic signatures of dissolved nitrate (NO_3) in water, for example, can help distinguish among nitrogenous wastes from different sources including agricultural runoff, animal waste groundwater contamination and municipal sewage discharges (Karr et al. 2002). Treatment of animal and human sewage that promotes volatilization of ammonia (e.g. alkaline conditions, high temperatures, and sewage holding ponds) causes characteristic fractionation of nitrogen pools. Because the lighter nitrogen isotope volatilizes more readily, measurable enrichment of the heavier isotope is found in the nitrogen remaining in the water and soil (Heaton 1986; Macko and Ostrom 1994; Showers et al. 1999). Similarly, oxygen isotopes in aqueous nitrates can be used to distinguish nitrate derived from industrial fertilizers (a solely atmospheric oxygen signature) from that derived from natural processes of nitrification (a mixture of water

and atmospheric oxygen signatures) (Aravena et al. 1993).

Isotopic compositions of inorganic (e.g. aqueous nitrates, δ^{15}N-NO_3) and organic (e.g. short-lived primary producers), representatives of the bottom tiers of the food web, can fluctuate substantially over time. Episodic pulses of non-point-source nitrogen pollutants often follow periods of concentrated fertilizer application and heavy rainfall (Showers et al. 1990). This punctuated delivery to the water column of runoff-derived pollutants can cause rapid shifts in water δ^{15}N-NO_3 signatures (e.g. over weeks or days). River discharge and groundwater recharge rates also affect dilution and transport of pollutants (Showers et al. 1990). Dilution of a pollutant in the water column does not, furthermore, necessarily correspond to removal of the pollutant from the environment. Nutrients are alternately retained and exchanged between biotic and abiotic components of an ecosystem. Nitrogen may enter the water via the atmosphere, soil or runoff, be incorporated into algae and bacteria by biological fixation (Webster and Ehrman 1996), be assimilated into animal tissues via the food web, and finally be recycled back to inorganic nitrogen in the water and soils via excretion or decomposition. As nutrients cycle between biotic and abiotic ecosystem components, they are carried intermittently downstream by the flow of water and the different movements of animals, plants, and substrates (Elwood et al. 1983). An immediate or direct relationship between nutrient influx and baseline biotic (whether producer or consumer) representation of nutrient and isotopic character cannot be assumed. Each compartment may play a different, and complementary, role in deciphering the extent and chronicity of background variation in isotopic character for community and habitat assessment.

The precedent for monitoring multiple environmental compartments has already been set in the field of toxicology, where bivalves serve as an important integrative sampling unit. Bivalves are used as biomonitors for a variety of lipophilic organic and inorganic pollutants (Cope et al. 1999), to complement traditional sediment and water analysis. Although the mechanisms of nitrogenous pollution compartmentalization relate to active uptake and assimilation by biota, rather than passive bioaccumulation of lipophilic toxicants, the justification for monitoring multiple environmental compartments is similar. Bivalve, and water or producer, isotopic signatures might complement each other by enabling chronological assessment of pollutant exposure. Dissolved nitrate signatures of water, sampled at daily to weekly intervals, capture the periodicity and range of baseline nutrient influx to freshwater systems (Showers et al. 1999). Bivalve signatures, in contrast, may provide a measure of the time-averaged persistence of nitrogenous nutrient pollutants in the aquatic environment.

The potential value of bivalves as integrative isotopic baselines for ecosystem studies, or sentinels of nutrient pollution, is clear. Confident use of bivalve signatures is, however, currently encumbered by limited understanding of:

1 the relationship between bivalve and ambient stable isotope compositions;
2 the correlation of isotope compositions between different types of bivalve tissue; and
3 the rate of change of bivalve compositions with time.

We explored the responsiveness of stable isotopic compositions of freshwater mussels to variations in ambient nutrient conditions, using nutrient-loading as a model of baseline shifts in isotopic character. In a series of field studies on the common freshwater unionid bivalve, *Elliptio complanata*, in streams in the Piedmont region of North Carolina we have investigated the variability and rate of change of bivalve stable isotopic compositions. The results have helped clarify the role of bivalves as biomonitors of nutrient loading, and, by extension, as isotopic baselines for ecological study in riverine systems.

Materials and method

Spatial variability

To assess baseline mussel δ^{15}N and δ^{13}C variability among sites and potential correlations with ambient water nitrate or particulate organic matter (POM) signatures, we sampled stream reaches adjacent to 22 road crossings in the Piedmont region of North Carolina (Fig. 1) between mid-May and early-July 2001 (Gustafson et al. 2005a). Riparian land cover at each location was predominately forested, with tree cover estimated at greater than two-thirds of the habitat adjacent to each stream. We surveyed a linear section of stream ranging from 300 m above to 300 m below each road crossing (Gustafson et al. 2005a) and collected 20 *E. complanata* from most (20) sites. Half of the animals were collected above the stream crossing at each of these sites, and the other half below the crossing. Only five *E. complanata* were collected from two sites because of limited availability. At these two sites, animals were collected where their availability was

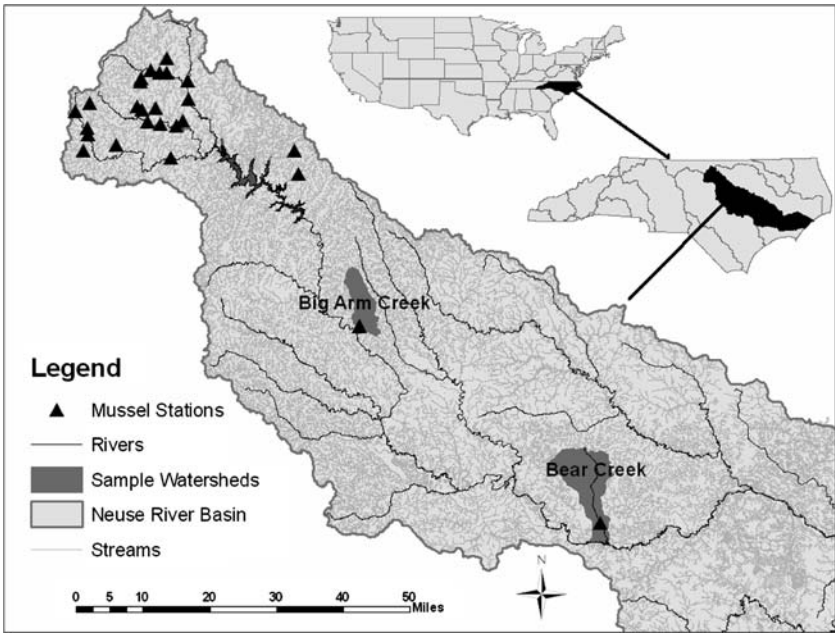

Fig. 1 Locations of 22 sampling stations in the Neuse River basin of North Carolina, USA for comparison of water, particulate organic matter, and freshwater mussel stable isotopic compositions

greatest (above the crossing at one site, and below at the other). A biopsy of foot tissue was taken non-lethally from each animal from each stream with ophthalmic surgical scissors through slightly open shell valves (Gustafson et al. 2005b). Animals were returned to their streams of origin directly after tissue collection. Tissue samples were placed in sterile microvials (Fisher Scientific, Pittsburgh, PA, USA), placed on ice for transport, and stored at −20°C in a freezer until $\delta^{13}C$ and $\delta^{15}N$ analysis. One-liter water samples were collected in acid-washed Nalgene containers (Fisher Scientific) from each stream (22 sites total). Comparative water samples were collected upstream and downstream of the road crossing from a convenience subsample of ten of these locations. All water samples were filtered through pre-combusted 0.47-μm GF/F filters (Fisher Scientific) on the day of collection, and both the filtered particulates (POM) and the filtrate (water) were stored at −20°C until $\delta^{13}C$ and $\delta^{15}N$ analysis.

Tissue type and sample size

Two of the streams with divergent baseline $\delta^{15}N\text{-}NO_3$ compositions were selected for more focused study of mussel $\delta^{15}N$ signatures. Big Arm Creek is a small tributary of the Neuse River near Clayton, North Carolina (Fig. 1). Its watershed is primarily forested and residential lands, and water $\delta^{15}N\text{-}NO_3$ signatures tend to be very low. Bear Creek is a larger tributary draining into the Neuse River near Goldsboro, North Carolina (Fig. 1). Its watershed includes drainage from several hog-waste lagoons, and water nitrate tends to be $\delta^{15}N$ enriched. Stream width and substrate material varied between these two sites (sandy substrate at Bear Creek, sand and gravel at Big Arm Creek), but they were chosen for their substantial differences in water signature. Five to ten *E. complanata*, and water samples for dissolved nitrate and POM analyses, were collected from each site five times over the course of a year, in September and November 2000 and January, May, and July 2001. Bivalves were scarce at the Bear Creek sampling site; consequently actual sample sizes varied with availability.

We collected and analyzed multiple sample types (foot tissue, whole hemolymph and serum-fraction hemolymph) from a convenience sample of 47 of these mussels (distributed from among the 10 different site-visits) to evaluate variation in $\delta^{15}N$ between sample-types. Bivalves have an open circulatory system: the circulatory fluid, called hemolymph, bathes tissues directly via large open spongy sinuses (McMahon and Bogan 2001) rather than diffusion through a complicated system of veins and arteries. Freshwater mussels have large hemolymph volumes for their size (McMahon and Bogan 2001), and this fluid is easily and safely collected from the sinus of the anterior adductor muscle, the surface of which is visible through slightly gaping valves (Gustafson et al. 2005b). Paired foot tissue and whole hemolymph samples were collected from all 47 animals. Serum-fraction hemolymph (cellular fraction removed from the liquid fraction) was also collected from a subset (29) of the 47 animals. Hemolymph was sampled from the anterior adductor muscle sinus, by tapping the muscle surface gently with a 25-guage needle and 1-cc syringe (Gustafson et al.

2005b). We collected approximately 0.5–1.0 cc of hemolymph from each mussel in sterile microvials, which were then placed on ice in the field and stored at −20°C until $\delta^{15}N$ analysis.

Inter-individual (population) variability can be substantial in certain lotic invertebrates (Lancaster and Waldron 2001). Reducing impacts on recovering or threatened freshwater mussel populations is a concern, however. We therefore used these site visits to estimate the precision of small sample sizes (five or six animals) and compared that with larger sample sizes (20 animals) taken during our stream survey. We used the coefficient of variation to describe the standardized variability of $\delta^{15}N$ values of different sample types and sizes (Lancaster and Waldron 2001).

Temporal variability and tissue turnover

We conducted additional sampling in Big Arm Creek (a total of 12 visits) over the course of 20 months (September 2000 through May 2002), to begin to characterize temporal variability in mussel signatures and to explore the stability of mussel and water signatures over time. At each interval we collected hemolymph from 5 to 10 resident *E. complanata* and water samples from approximately the same 3-m section of the stream. The subset of concurrent visits to Bear Creek, described above, revealed consistent divergence in water and mussel isotopic compositions between the two sites.

In May 2001 we collected six animals from Bear Creek (the study site with characteristically enriched mussel $\delta^{15}N$ and dissolved $\delta^{15}N$-NO_3 signatures) and transported them to Big Arm Creek. Our goal was to monitor signatures of five transplant animals for 1 year; an extra animal was included to compensate for potential mortality. All six animals survived, however, so data from all six were retained in the study. At Big Arm Creek the transplants were placed in a plastic mesh box enclosure (30 cm × 30 cm × 46 cm) with the bottom lined with sediment and gravel from the stream bed. The box enclosure was secured to a fallen tree and placed on the stream bed at a depth of approximately 0.6 m. The site was visited in June, July, August, and October 2001, and January, March, and May 2002 to collect hemolymph from the six transplant animals and five free-ranging animals native to Big Arm Creek for $\delta^{15}N$ evaluation. At each visit we collected a water sample for filtration and water $\delta^{15}N$-NO_3 and particulate organic $\delta^{15}N$ analysis. We targeted whole hemolymph for this translocation study because repeated collection is non-lethal (Gustafson et al. 2005b), much less invasive than tissue biopsy, and does not depend upon field access to a centrifuge. Duplicate hemolymph samples were collected from one of the cohorts of six mussels to evaluate sampling variability.

Isotopic analysis

Hemolymph and foot tissue samples were freeze-dried for $\delta^{15}N$ and $\delta^{13}C$ analyses. Water was prepared for nitrate $\delta^{15}N$ analysis by double ion exchange (Chang et al. 1999). Prepared samples were combusted in a Carlo Erba NC 2500 elemental analyzer and the N_2 peak was injected into a Finnegan Mat Delta + XLS continuous flow isotope ratio mass spectrometer (CF-IRMS). The nitrate $\delta^{15}N$ of the water and the $\delta^{15}N$ of the bivalve tissues are reported, using δ notation, in per mil (‰) deviations from atmospheric nitrogen using the convention: $\delta^{15}N$ (‰) = $[(^{15}N{:}^{14}N_{sample}/^{15}N{:}^{14}N_{atmN2}) - 1] \times 10^3$. The $\delta^{13}C$ of bivalve tissues and coarse particulate organic matter was analyzed in a similar fashion. Results are reported in per mil (‰) deviations from PeeDee Limestone using the convention: $\delta^{13}C$ (‰) = $[(^{13}C{:}^{12}C_{sample}/^{13}C{:}^{12}C_{PDB}) - 1] \times 10^3$.

Statistical analysis

Parametric distributions of $\delta^{15}N$ and $\delta^{13}C$ values were confirmed using the Anderson–Darling test (Steel et al. 1997). Mean bivalve foot isotopic signatures were compared with those of corresponding site water. We used the paired *T* test to detect differences between above-bridge and below-bridge sampling locations, and between duplicate samples, and used simple linear regression to evaluate associations between tissue types (Steel et al. 1997). To assess the importance of sample size in accurate estimation of a population mean, we calculated variation within sample sets of different size using the CV (coefficient of variation expressed as a percentage; Lancaster and Waldron 2001). Temporal tracking of water versus mean bivalve hemolymph signatures was also described using simple linear regression (Steel et al. 1997), and temporal variability is demonstrated with graphs and descriptive statistics. The tissue turnover time, or half-life, of hemolymph was calculated as $T_{1/2} = (\ln 2)/k$ (Hobson and Clark 1992; Six and Jaystrow 2002), where k is the absolute value of the $\delta^{15}N$ depletion rate. The depletion rate is typically calculated from the exponential equation $y = a + be^{kt}$, where $y = \delta^{15}N$ of the bivalves at time t after transplant, a is the asymptotic isotopic signature (e.g. that of the resident Big Arm population), and b is the difference between transplant and resident signatures at start (Hobson and Clark 1992). Because we could not assume a stable asymptotic endpoint,

Table 1 Average (of site mean) isotopic values for freshwater mussels, water NO_3, and particular organic matter (POM)

Isotope	Sample	*N* (sites)	Range (‰)	Mean (‰)	SD (‰)
$\delta^{15}N$	Foot	22	(4.94, 9.125)	7.516	1.018
	Water NO_3	16	(2.865, 14.589)	6.458	2.848
	Water POM	16	(0.833, 7.192)	4.452	1.675
$\delta^{13}C$	Foot	20	(−31.7, −25.149)	−28.281	1.648
	Water POM	16	(−30.952, −25.298)	−27.875	1.646

Sample sizes for mussel foot tissue varied from 5 to 20 individuals per site. Water and POM signatures represent either single samples (for 12 sites) or averages for duplicate samples (for 10 sites)

Table 2 Mean isotopic values were different, although the differences were not statistically significant, for upstream and downstream sampling locations

Sample	*N* (sites)	Upstream		Downstream		*t*	*P* value
		Mean (‰)	SD (‰)	Mean (‰)	SD (‰)		
Bivalve foot $\delta^{15}N$	20	7.567	0.999	7.504	0.990	0.45	0.657
Bivalve foot $\delta^{13}C$	20	−28.375	1.608	−28.190	1.721	−1.94	0.067
$\delta^{15}N$-NO_3	10	5.75	3.69	6.86	3.13	−1.51	0.164

The average value from foot samples from ten mussels per location was used to represent each site. Site water NO_3 signatures are represented by single samples

however (Big Arm signatures were not constant over time), we altered the equation to model the diminishing difference, z, between the transplant and resident populations over time t. In this situation, the asymptote a (the difference at equilibration) is zero, and the equation reduces to $z = be^{kt}$. The term k was estimated as the slope of the regression line of $\ln(z/z_0)$ versus time, where z_0 was the original difference between Bear and Big Arm signatures on day 0 of the transplant. We included data from the date of translocation to the end of January, when transplant signatures first closely approximated resident signatures (Fig. 3).

Results

Spatial variability

Foot tissue of bivalves from 22 stream reaches displayed significant spatial variability in $\delta^{15}N$ (ANOVA $P < 0.001$) and $\delta^{13}C$ (ANOVA $P < 0.001$) isotopic composition by site and was matched by similar or more extensive variation in dissolved $\delta^{15}N$-NO_3 and POM (particular organic matter) $\delta^{15}N$ and $\delta^{13}C$ values (Table 1). Mean bivalve foot $\delta^{15}N$ values were similar between upstream and downstream locations (Table 2). Delta ^{15}N-NO_3 and $\delta^{13}C$ values were consistently higher, on average, below a road crossing than above, although neither was statistically significant at an α of 0.05 (Table 2). Thus, we grouped animals and water samples by stream reach, and did not differentiate between upstream and downstream locations, in remaining analyses. Site mean bivalve foot $\delta^{15}N$ values correlated with ambient water $\delta^{15}N$-NO_3 (bivalve foot $\delta^{15}N = 6.56 + 0.159\delta^{15}N$-$NO_3$) but not ambient POM $\delta^{15}N$ (Table 3). POM $\delta^{13}C$ values, in contrast, were predictive of mean bivalve $\delta^{13}C$ composition (bivalve foot $\delta^{13}C = -8.33 + 0.712$POM $\delta^{13}C$) (Table 3).

Table 3 Bivalve isotopic values correlated with those of stream water and POM

Sample 1	Sample 2	*N* (sites)	R^2 (%)	*P* value
Bivalve foot $\delta^{15}N$	Water $\delta^{15}N$-NO_3	16	27.6	0.037
	POM $\delta^{15}N$	16	0.1	0.921
Bivalve foot $\delta^{13}C$	POM $\delta^{13}C$	16	49.5	0.002

Sample sizes for mussel foot tissue varied from 5 to 20 individuals per site. Site water and particulate organic matter (POM) values are represented by single samples or averages from duplicate samples

Tissue types and sample sizes

Bivalve hemolymph $\delta^{15}N$ values correlated well with those of corresponding foot tissue. Linear regression analyses yielded an R^2 value of 79.2% (foot = 1.19 + 0.86 whole hemolymph) for foot to whole hemolymph comparisons ($P = 0.000$, $n = 47$ mussels), and an R^2 value of 75.4% (foot = 1.83 + 0.81 serum) for foot to serum-fraction hemolymph comparisons ($P < 0.001$, $n = 29$ mussels). On average, foot tissue was more ^{15}N enriched than whole hemolymph and serum.

Mean coefficients of variation (CV) from individual location–date cohorts were similar among samples of different size, and $\delta^{15}N$ values of duplicate hemolymph samples were not statistically different. The mean CV for foot $\delta^{15}N$ was 5.4% (range 3.6–8.4, $n = 5$ cohorts from two streams) and 5.7% (range 3.0–10.0, $n = 3$ cohorts from two streams) for sample sizes of five and six animals, respectively. Similarly, the mean CV for whole hemolymph from individual location–date cohorts was 4.4% (range 1.3–8.0, $n = 10$ cohorts from two streams) for a sample size of five animals. In comparison, sample sets of 20 animals (although from a different set of streams) yielded a mean CV for foot $\delta^{15}N$ of 6.6% (range 3.7–21.4, $n = 19$ stream sites).

There were no significant differences between $\delta^{15}N$ values for duplicate hemolymph samples ($n = 6$ duplicates, paired $T = 0.29$, $P = 0.783$). The mean absolute difference between duplicate signatures was 0.08‰ (standard deviation = 0.678‰).

Temporal variability

Within the confines of a single stream, mean bivalve hemolymph signatures did not correspond to fluctuations in point-in-time water $\delta^{15}N$-NO_3 values ($P = 0.49$, $R^2 = 4.8\%$, $n = 12$ visits) or point-in-time POM signatures ($P = 0.59$, $R^2 = 3.0\%$, $n = 12$ visits). Water and POM signatures were much more variable over time than corresponding mean bivalve signatures (Fig. 2). Big Arm Creek was visited 12 times between September 2000 and May 2002. During that period, mean bivalve signatures averaged 6.37‰ (SD 0.56‰), with a coefficient of variation (CV) of 8.8%. Water $\delta^{15}N$-NO_3, in contrast, averaged 4.98‰ (standard deviation 1.31‰) with a CV of 26.3%, and POM $\delta^{15}N$ averaged 4.25‰ (SD 1.84‰) with a CV of 43.3%.

Tissue turnover

The six mussels transplanted from Bear Creek to Big Arm Creek had $\delta^{15}N$ values that were consistently more ^{15}N enriched than the Big Arm Creek natives (Fig. 2). Over the course of the summer slow equilibration of $\delta^{15}N$ values with those of native animal signatures was observed, and by January, the difference between the $\delta^{15}N$ signatures of the transplants and natives had narrowed substantially (Fig. 3). Isotopic differences between transplant and native bivalves were statistically significant (T test $P < 0.05$) in May, June, late August, and October 2001, and again in May 2002. Tissue signatures of the transplants first closely approximated those of the native animals at the end of January 2001. A regression of $\ln(z/z_0)$ over time

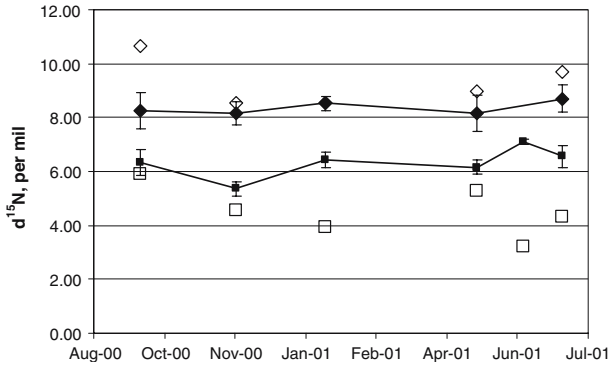

Fig. 2 $\delta^{15}N$ of bivalve whole hemolymph and water NO_3 of two streams over time. *Error bars* represent means ±1 SD ($n = 4$–6) for bivalve signatures. Water values shown are single data points. Symbols for mussel means are *solid* and connected by *lines* (one for each stream). Symbols for water values are *open*. *Squares* represent samples taken from Big Arm Creek. *Diamonds* represent samples taken from Bear Creek

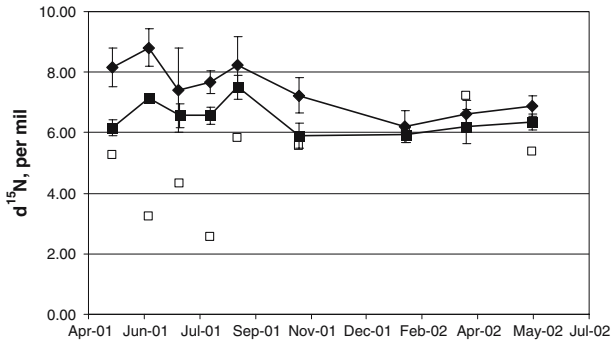

Fig. 3 $\delta^{15}N$ of Big Arm Creek natives, transplanted mussels, and water NO_3 over time. *Circles* and *squares* with *error bars* represent mean values ±1 SD for bivalve whole hemolymph $\delta^{15}N$ ($n = 4$–6 per cohort). Water values shown are single data points. Symbols for mussel means are *filled-in* and connected by *solid lines* (one for each creek). Symbols for water values are *open*. *Squares* represent samples taken from Big Arm Creek. *Diamonds* represent samples taken from Bear Creek

resulted in an R^2 of 69.3% ($P = 0.02$). The fitted regression, $\ln(z/z_0) = 0.08 - 0.00613t$, where t is days from start, provided an estimate of k of 0.00613. This resulted in an estimate for hemolymph turnover time, $T_{1/2} = -\ln(0.5)/k$ (Hobson and Clark 1992; Raikow and Hamilton 2001; Six and Jaystrow 2002), of 113 days.

Discussion

Our study clarifies the suitability of freshwater mussels as isotopic baselines for riverine communities. The survey of *E. complanata* from 22 streams in North Carolina corroborates other studies demonstrating spatial stratification of bivalve stable nitrogen and carbon isotope signatures by site (McKinney et al. 1999;

Post et al. 2000) or land-use (Fry 1999; McKinney et al. 2002) characteristics. We found a positive association between dissolved δ^{15}N-NO_3 and corresponding mussel tissue signatures across sites, consistent with the speculated relationship between bivalve δ^{15}N composition and nutrient loading in ambient waters (Cabana and Rasmussen 1996; Hansson et al. 1997; Kwak and Zedler 1997; McClelland and Valiela 1998). Although the association between resident mussel signatures and a site's water signature was statistically significant, the two compartments were not tightly correlated. Our range of dissolved δ^{15}N-NO_3 signatures was much wider than our range of average mussel δ^{15}N signatures from the 22 sampling sites, with a dampening, or time-averaging, of signature response from water to bivalve.

Similarly, within the confines of a single stream, mean bivalve hemolymph signatures did not closely track temporal fluctuations in dissolved δ^{15}N-NO_3 signatures. The lack of tight temporal coupling between water and mussel isotopic compositions points again to the relative volatility of dissolved δ^{15}N-NO_3, one of the liabilities of water and phytoplankton-based sampling efforts. Dissolved inorganic nitrogen signatures in water respond rapidly to episodic nutrient inputs (Karr et al. 2002), shaped by stream discharge rates, groundwater recharge, and storm-water runoff (Showers et al. 1990). Consequently, the frequency of water or particle-based sampling necessary to reliably capture peaks and troughs in isotopic character varies by river and by season, is costly and time-consuming (Showers et al. 1999; Showers WJ, Bucci J, Gustafson L, Levine J, Bogan A, Lewis T, Taylor S., Isotopic determination of growth rates in freshwater mussels (Bivalvia: Unionidae *Elliptio complanata*), unpublished data). Although not intensive enough to fully characterize the fluctuations of δ^{15}N-NO_3 in river water, our longitudinal sampling intervals (measured in months) did provide further evidence of the clear and important differences between the volatility of dissolved δ^{15}N-NO_3 and tissue δ^{15}N compositions. The CV, an index of relative dispersion, was three times greater for stream water δ^{15}N-NO_3 samples collected from September 2000 to May 2002 than it was for bivalve hemolymph signatures collected concurrently.

Results from our transplant study provide further evidence that the isotope composition of bivalves responds to changes in environmental signatures, although slowly. Transplanted animals equilibrated to the water δ^{15}N-NO_3 signatures of their new environment at a rate of 0.006‰ change per day. Although monitoring efforts based on dissolved δ^{15}N-NO_3 or particulate organic material are often tailored to weekly or daily intervals (Showers et al. 1999; Post et al. 2000; Showers et al. manuscript in preparation), monthly or seasonal sampling intervals seem sufficient for mussel tissues. Consequently, bivalve signatures do provide general estimates of water δ^{15}N-NO_3 and POM signatures at the base of the food web, although they are not suitable proxies for detecting pulse events or sudden shifts in dissolved inorganic nitrogen loads. Rather, by responding slowly to ambient signatures, bivalves are dampened integrative monitors of chronic, or averaged, nutrient conditions. They seem to be a relatively stable and time-integrated estimate of general baseline isotopic character. Consequently, tandem evaluation of water (or a short-lived primary producer) and bivalve isotopic signatures would offer, rather than redundancy, a selection of complementary vantage points for evaluation of both long and short-term effects of nutrients.

Understanding the time required, and pathways represented, for incorporation of an isotopic change in ambient signatures is critical for ecological studies. Disturbance of the baseline may register among different food web constituents at different rates depending upon their trophic position, metabolism, and designated use of the nutrients. Our study demonstrates a relationship between isotopic signatures of dissolved nitrogenous inorganic compounds and the tissues of freshwater mussels, although details of the trophic pathways for incorporation of these nutrients remain in question. It is interesting to note that the isotopic composition of bivalve nitrogen did not correlate statistically with that of ambient POM in either the spatial or temporal surveys. Although average differences between bivalve and POM ^{15}N approximated the 3–4‰ mean trophic fractionation between diet and consumer (Nichols and Garling 2000; Post 2002), the lack of a statistically significant correlation between bivalve and POM ^{15}N calls into question whether the bivalve diet is entirely algae-based. In contrast, bivalve ^{13}C in our study did correlate with spatial variation in ^{13}C of ambient POM. Bivalve ^{13}C (from foot tissue), however, was, on average, 0.4‰ more depleted than ambient POM: a value lower than the 0–1 average trophic enrichment estimated by earlier studies (Nichols and Garling 2000; Post 2002). Though muscle and hemolymph are relatively low in lipids (McMahon and Bogan 2001; McCutchan et al. 2003), lipids were not removed from our foot samples, so may account for some of the relative depletion (Focken and Becker 1998). Bivalve isotopic signatures have been shown elsewhere to be more enriched in nitrogen and less so in carbon than expected from a diet of, predominantly, algae (Nichols and Garling 2000; Lorraine et al. 2002), however, and a

conventional algal diet does not seem to be sufficient for growth of captive adult unionids (Gatenby et al. 1999; Naimo et al. 2000). A growing body of evidence suggests that algae are only part of a much more complex diet that probably includes bacteria (McMahon and Bogan 2001; Nichols and Garling 2000), detritus (McMahon and Bogan 2001; Raikow and Hamilton 2001), primary consumers (Jiffry 1984), and perhaps even soluble organics (Roditi et al. 2000). Consequently, ecological food web studies based on freshwater mussel signatures need to consider whether the baseline represented is entirely algae-based. Concurrent evaluation of more than one baseline constituent (e.g. a primary producer and primary consumer), and at multiple points in time, will enable better resolution of the isotopic stability of the system under consideration and improve resulting confidence in ecological descriptions and predictions.

Non-lethal collection of hemolymph is a relatively simple procedure (Gustafson et al. 2005b) and seems a suitable alternative to the more-invasively sampled foot tissue for estimation of $\delta^{15}N$ composition. Our calculations of inter-population variability (Lancaster and Waldron 2001) for *E. complanata* reveal the CV (5%) is reasonable for foot tissue and whole hemolymph in cohort sizes of five and six animals, suggesting that relatively small sample sizes may provide reasonable estimates of a population mean. Hemolymph may, furthermore, be one of the tissues to respond earliest to habitat change. The tissue turnover time of hemolymph of *E. complanata* was slightly slower than the response time of stomach gland and gut contents (78 and 85 days, respectively) but faster than that of muscle tissue (357 days) described previously for the freshwater bivalve *Pleurobema* sp. (Raikow and Hamilton 2001), and faster than that of whole-body tissue (333 days) described for the marine mussel *Mytilus edulis* (Hawkins 1985). The focus of ecological study may dictate choice of sample type. For example, a tissue with a relatively short half-life might best detect seasonal or land-use change whereas longer half-life tissues might be preferred for the estimation of trophic relationships. Effect on the sampled population is another important consideration, however. We chose to monitor hemolymph for its relative safety and ease of collection compared with the more common but more invasive foot and mantle tissue samples. When choice of tissue type is flexible, hemolymph is a suitable, and less invasive, alternative to traditional sampling.

As temporal integrators of background fluctuations in source carbon and nitrogen signatures, long-lived primary consumers have great potential as isotopic baselines for ecological study (Post 2002). Our research helps to clarify temporal and spatial variability in stable isotopic compositions of freshwater mussels and, consequently, their role as isotopic baselines in freshwater riverine systems. Isotopic compositions of freshwater mussels varied over both space and time, but much less dramatically than that of water nitrates or POM. In addition, isotopic response to spatial relocation was definitive, with a hemolymph turnover time estimated at 113 days. Mussel compositions better reflected the $\delta^{15}N$ of water nitrates than algae, however, and were lower in $\delta^{13}C$ than a pure algal diet might predict, questioning whether these filter-feeders have a more varied food base. To avoid unnecessary handling of bivalves from imperiled populations, and to ensure comparable results for longitudinal study, hemolymph sampling for stable isotopic determination should target known stable (for example *Elliptio complanata*) populations or invasive (e.g. *Corbicula*) species. Where freshwater bivalve populations are abundant and stable, however, non-lethal assessment of bivalve hemolymph compositions can add an important dimension to nutrient-loading and ecosystem studies alike.

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