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Eastern oyster (*Crassostrea virginica*) δ^{15}N as a bioindicator of nitrogen sources: Observations and modeling

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ABSTRACT

Stable nitrogen isotopes (δ^{15}N) in bioindicators are increasingly employed to identify nitrogen sources in many ecosystems and biological characteristics of the eastern oyster (*Crassostrea virginica*) make it an appropriate species for this purpose. To assess nitrogen isotopic fractionation associated with assimilation and baseline variations in oyster mantle, gill, and muscle tissue δ^{15}N, manipulative fieldwork in Chesapeake Bay and corresponding modeling exercises were conducted. This study (1) determined that five individuals represented an optimal sample size; (2) verified that δ^{15}N in oysters from two locations converged after shared deployment to a new location reflecting a change in nitrogen sources; (3) identified required exposure time and temporal integration (four months for muscle, two to three months for gill and mantle); and (4) demonstrated seasonal δ^{15}N increases in seston (summer) and oysters (winter). As bioindicators, oysters can be deployed for spatial interpolation of nitrogen sources, even in areas lacking extant populations.

1. Introduction

Measurements of stable nitrogen isotopes (δ^{15}N) are increasingly used to identify nitrogen inputs from anthropogenic sources; agricultural runoff as well as human and animal wastes (Costanzo et al., 2001; Kendall, 1998; McClelland et al., 1997). Many examples suggest the suitability of δ^{15}N in many organisms for this purpose, including macrophytes (e.g. Benson et al., 2008; Cohen and Fong, 2006; Cole et al., 2004; McClelland et al., 1997), finfish (Lake et al., 2001; Schlacher et al., 2005), and mollusks (Fila et al., 2001; McKinney et al., 2001, 2002), or some combination (Fry et al., 2003; Gartner et al., 2002). Enriched δ^{15}N signatures in sewage or animal waste arise from isotopic discrimination due to a combination of ammonia volatilization and denitrification at the source or by microbial processing employed by wastewater treatment facilities (Fry, 2006; McClelland and Valiela, 1998; Sweeny and Kaplan, 1980; Tucker et al., 1999). In contrast, synthetic fertilizers are 'fixed' from atmospheric N_2 (0‰) and have corresponding δ^{15}N values: generally −4‰ to +4‰ (Hübner, 1986; Macko and Ostrom, 1994; Vitoria et al., 2004).

Terrestrial nitrogen sources may have complicated pathways before reaching aquatic biological indicators, and isotopic signatures can be modified or mixed en route, therefore interpretations must consider alternative hypotheses (Fry, 2006; Kendall, 1998). Nitrogen from animal manures, for example, can be fractionated during volatilization which favors ^{14}N and enriches the remaining nitrogen pool with ^{15}N (Altabet, 2006; Cline and Kaplan, 1975; Fry, 2006; Kendall, 1998; McClelland and Valiela, 1998), and dissolved nitrate can be microbially denitrified to gaseous N_2, elevating the remaining nitrate pool (Mariotti et al., 1982; Shearer and Kohl, 1988). Once entering estuarine waters, dissolved inorganic nitrogen is assimilated by phytoplankton and subsequently consumed by oysters with an accompanying enrichment of 3–4‰ at each trophic step as fractionation occurs during digestion and waste elimination (Adams and Sterner, 2000; Minagawa and Wada, 1984). Further, δ^{15}N signatures may vary due to metabolic factors including nutritional stress (Fuller et al., 2005; Hobson et al., 1993), weight change, life stage (Grant and Kopple, 2009), gestation (Fuller et al., 2005), and starvation (Boag et al., 2006; Haubert et al., 2005; Hobson et al., 1993).

Measuring δ^{15}N in biological indicator species, particularly bivalves such as the eastern oyster (*Crassostrea virginica*), can be advantageous over direct measurements made on nitrogen in groundwater (Aravena et al., 1993; Jin et al., 2004; Lefebvre et al., 2007), the water column (Cole et al., 2006), or sediments

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(Tucker et al., 1999), as oysters integrate spatial and temporal variability. Bivalves indicated a variety of contaminants (Bebianno et al., 2004; Carmichael et al., 2008; Kimbrough et al., 2008) including nitrogen sources (Daskin et al., 2008; Fila et al., 2001; McKinney et al., 2002; Moore and Suthers, 2005), and δ^{15}N in filter feeders has been compared to that in primary producers as bioindicators of nitrogen sources (Fertig et al., 2009; Fry et al., 2003). In Chesapeake Bay, oysters' historic prevalence (Newell, 1988) and ecological roles (Kemp et al., 2005) make this species an ideal bioindicator, and additionally oysters are sessile, hardy, euryhaline, and tolerant to transplantation (Powell and Ashton-Alcox, 2004) enabling deployment in numerous locations for spatial assessments. Further, oysters assimilate nitrogen from suspended particulate organic matter (phytoplankton, other microbes, as well as detritus; Langdon and Newell, 1996) throughout their lives, and do not substantially change diets post-metamorphosis or with developmental stages (i.e. ontogenetic shifts). Therefore, the length of usefulness is not limited by life cycle patterns, in contrast to some species of arthropods (Haubert et al., 2005), crustacean zooplankton (Ventura and Catalan, 2008), and estuarine fish (Griffin and Valiela, 2001; Witting et al., 2004).

Knowledge gaps about stable isotopes in organisms persist, including the influence of variables such as locality, tissue type, seasonality, and temporal integration and are addressed in this work. Convergence of δ^{15}N to new conditions was observed experimentally after diet changes (Adams and Sterner, 2000) and post-deployment in the field (Daskin et al., 2008; Dattagupta et al., 2004), but not previously for *C. virginica* in a water body with a large, multi-use watershed. To account for δ^{15}N variation in *C. virginica*, a baseline was established through fieldwork and modeling exercises to investigate oyster muscle, gills, and mantle in areas of Chesapeake Bay. Specifically, the following research topics were addressed: (1) optimization of sample size; (2) verification that δ^{15}N in oysters from two locations converged to water column δ^{15}N values after shared deployment in new locations; (3) identification of required exposure time and duration of temporal integration; and (4) assessment of seasonal variations.

2. Methods and materials

2.1. Study location

Fieldwork was conducted in Monie Bay and the South, Severn, and Choptank Rivers (Fig. 1a–d) which are tributaries in the mesohaline region of Chesapeake Bay (Chesapeake Bay Program, 2004). Measurements of standard water quality metrics (salinity, temperature, and dissolved oxygen concentration and saturation) in these regions were conducted using a standard YSI 85 handheld dissolved oxygen and conductivity instrument (YSI Inc.), and Secchi depth was also measured.

2.2. Isotope analyses

Upon collection, oysters were kept on ice until frozen (−20 °C) at the laboratory until preparation for isotopic analysis. For preparation, oysters were thawed, shell height (mm) was measured with calipers, and individuals were dissected to obtain the adductor muscle, gills, and mantle. Tissues were rinsed to avoid contamination by carbonates from the shell and then oven dried (60 °C) until completely dry (48 h minimum). Dried tissues were ground and homogenized by mortar and pestle (see Coleman and Fry, 1991; Knowles and Blackburn, 1993). Tissue sub-samples (1.0 ± 0.2 mg) were packed into tin capsules (Elemental Microanalysis, pressed, standard weight, 8 × 5 mm).

Where specified, seston (60 ml) was also collected on pre-combusted glass fiber filters (25 mm Wattman GF/F) for δ^{15}N. Filters were packaged in tin foil and kept on ice in the field and frozen in the laboratory until preparation for analysis. Filters were thawed, thoroughly oven dried (60 °C), rolled, then pressed into tin foil discs for isotopic analysis. Nitrogen content (μg N) and δ^{15}N were analyzed for oysters and seston by the University of California Davis Stable Isotope Facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Calculations of molecular %N in oysters were also conducted to assess nutritional

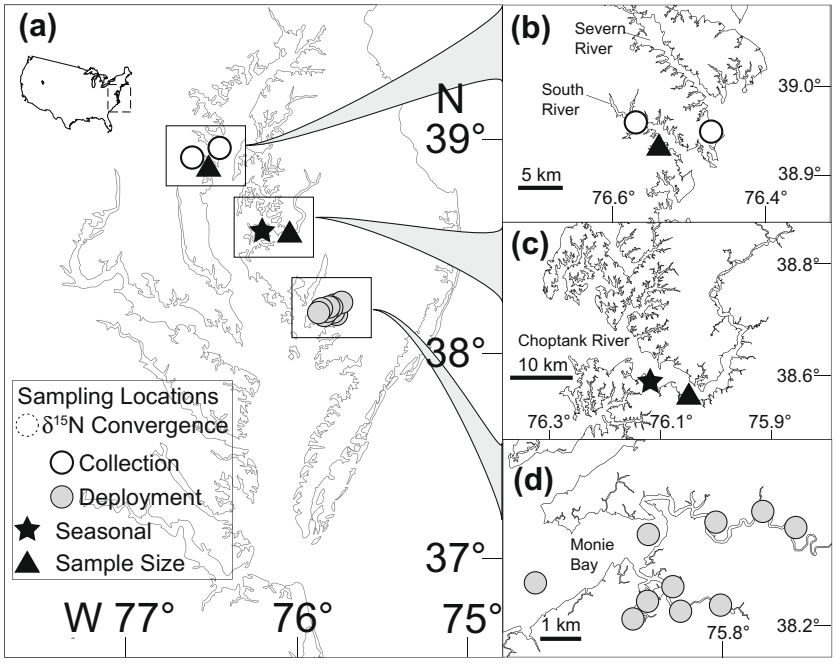

Fig. 1. Map of regions within Chesapeake Bay studied (a). White circles indicate collection sites in the South and Severn Rivers (b) for deployment (grey circles) in Monie Bay (d). Black triangles indicate collection sites in the South and Choptank Rivers for the sample size determination (b and c). The black star (c) indicates where seasonal variations were observed in the Choptank River.

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condition based upon the molar ratio of the sub-sample nitrogen content (μg) to sub-sample dry weight (mg). For definition, $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 10^3$, where R was defined as the $^{15}N/^{14}N$ ratio. The standard reference was atmospheric N_2 (air), with 0.3663 at% ^{15}N, defined as 0‰ (e.g. Fry, 2006) and instrumental error was ±0.2‰.

Isotopic analysis on dissolved inorganic nitrogen was conducted on 125 ml filtered water column samples collected in May and June 2009 using the methods of NO_3^- reduction to NO_2^- from McIlvin and Altabet (2005) and NH_4^+ oxidation to NO_2^- from Zhang et al. (2007) followed by reduction of NO_2^- to N_2O through a reaction with azide. These methods require very low nitrogen sample size (15 nmol). For the McIlvin and Altabet (2005) method, Cd powder used for NO_3^- reduction was prepared as a slurry with deionized water and 10% HCl, which was added drop-wise and mixed with an Aerolatte™ coffee stirrer. Addition of acid continued until the cadmium lost its brown color and the overlying liquid was clear. Care was taken to avoid agglutinization. Modifications from Zhang et al. (2007) included use of 4 ml of the BrO^- working solution for a 20 ml of sample, immediate vigorous shaking for 30 s, 1 h reaction time, and 0.8 ml arsenite reagent to stop the reaction. Correspondingly, 0.8 ml 6 N HCl was added to a 5 ml aliquot to lower pH sufficiently for the colorimetric determination of concentration (indophenol method, Strickland and Parsons, 1972) of NO_2^- after oxidation. For both NO_3^- reduction and NH_4^+ oxidation, 0.87 mg NaCl was added to bring chlorinity to that of seawater for samples with salinity <5 (Millero, 2005). Dissolved N_2O gas was then analyzed on an isotope ratio mass spectrometer. Reproducibility of $\delta^{15}N$ values was ±0.5‰.

2.3. Determination of optimal sample size

Oysters were collected randomly from the Choptank River (38°34′19″N, 76°3′14″W) on 14 June 2005 and from the South River (38°55′51″N, 76°32′16″W) on 17 June 2005 (Fig. 1a–c). Variability of standard errors was assessed to optimize the trade-off between resolving power and expended effort using methods described by Bros and Cowell (1987). The standard error of the deviations for $\delta^{15}N$ data was calculated for various sample sizes (2–20) which were randomly selected 10 times for each tissue, and the mean, maximum, and minimum standard error of the deviations of these selections was plotted against sample size.

2.4. Verification of convergence after shared deployment

A transplantation study was conducted to verify that $\delta^{15}N$ in oysters from two locations converged towards a common $\delta^{15}N$ value after shared deployment. Oyster spat on shell were transplanted (Fig. 1a, b and d) in June–October 2006 from two Chesapeake Bay tributaries (the South River: 38°57′11″N, 76°34′21″W and the Severn River: 38°56′42″N, 76°28′1″W) to 10 stations in Monie Bay and its three creeks (38°13′30″N, 75°50′00″W). Two mesh (3.61 cm^2 holes) bags were deployed at each of the 10 stations such that both source tributaries were represented by a mesh bag at each of the ten sampling stations (Fig. 1a, b and d). Mesh bags contained 20 oysters each, from their respective sources, and were anchored with three bricks and suspended 0.5 m off the bottom using a marked buoy to minimize sediment smothering. Five randomly selected surviving oysters (23% mortality rate from 100% recovery of bags) from each source tributary were sampled for isotopic analyses. The oysters were in apparent good health based on observations of new growth and stable nutritional condition (%N). Fouling organisms and trapped sediments were removed from mesh bags fortnightly or as needed to maintain water flow through the mesh bags.

Multiple assessments were made to quantify the convergence of oyster $\delta^{15}N$ values after deployment and to verify that the post-deployment $\delta^{15}N$ values resembled isotopic signatures of nitrogen sources at the location of deployment. Post-deployment $\delta^{15}N$ values in oysters from the Severn River were compared and regressed to post-deployment $\delta^{15}N$ values in oysters from the South River (Proc Reg, SAS Institute) and regression slopes were compared to the 1:1 line and to each tissue (Proc GLM, SAS Institute). Additionally, the convergence of oyster $\delta^{15}N$ was modeled (based on inputs of measured seston $\delta^{15}N$ values) and compared to measured $\delta^{15}N$ values, as described below, to identify acclimatization patterns over seasons and a range of environmental conditions and seston $\delta^{15}N$ values. Dissolved inorganic nitrogen $\delta^{15}N$ values were compared to seston and oyster tissue $\delta^{15}N$ values to verify that each reflected ambient conditions in the water column based on the assumption that most available nitrogen is assimilated by seston and oysters. Isotopic fractionation varies widely (Wada et al., 1975; Hoch et al., 1992; Montoya and McCarthy, 1995; Waser et al., 1998; Pennock et al., 1996) due to algal assimilation of nitrate (+1‰ to +19‰), nitrite (−9‰ to +4‰) and ammonium (−7‰ to +2‰), and can be affected by nitrogen concentrations and algal species. Expected $\delta^{15}N$ values were generated by adding 3–4‰ per trophic level (i.e. seston assimilation by oysters; Minagawa and Wada, 1984) to dissolved inorganic $\delta^{15}N$ values. A trophic fractionation of 3–4‰ is reasonable due to mesohaline salinities, likely dominance of flagellate algal species, and low nutrient concentrations in Monie Bay (Wada et al., 1975; Montoya and McCarthy, 1995; Waser et al., 1998). To account for varying temporal integrations among tissues, expected oyster muscle tissue $\delta^{15}N$ values were adjusted by +1.0‰.

2.5. Quantifying temporal integration and required exposure time

To identify temporal patterns of isotopic signatures and assess integration by $\delta^{15}N$ values in oyster tissues, periodic measurements of seston $\delta^{15}N$ values and other water quality metrics were conducted in Monie Bay and the Choptank River. In Monie Bay (Fig. 1a and d), seston $\delta^{15}N$ values were measured six times during the four month deployment period, and means of each time period combination were regressed against oyster tissue $\delta^{15}N$ values. Additionally, $\delta^{15}N$ values from each tissue were assessed to identify if tissues integrated over different periods of time. In the Choptank River (38°35′36″N, 76°07′43″W, Fig. 1a and c), variations of salinity, temperature, dissolved oxygen, total nitrogen, total phosphorus (three replicate 20 ml nutrient samples), seston (three 60 ml replicates collected and analyzed for isotope values as described above), and oyster $\delta^{15}N$ (mantle, gills, and muscle tissues from five individuals) were observed monthly over two years. Total nutrient concentrations were analyzed by standard methods (D'Elia et al., 1977; Kerouel and Aminot, 1987).

Required exposure times, seasonal oyster $\delta^{15}N$ values, and optimal timing of deployment were inferred by modeling the incorporation of seston $\delta^{15}N$ into oyster tissues. The model assumed the tissue in question (mantle, gills, or muscle) responded due to a temperature dependent tissue turnover, which reflected a mixture of the seston isotopic signal encountered (model input values) as well as a trophic shift (a constant 3.0‰). Instantaneous scaled temperatures were calculated by interpolating temperature measurements between each time step and then normalizing the temperature range between 1 (maximum in summer) and 0 (minimum in winter). In Monie Bay and its creeks, the winter temperature minimum was assumed to be ~0 °C (Apple et al., 2006).

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Specifically, each tissue was modeled according to the equation:

$$\delta_O(t) = \delta_O(t - \Delta t) - \Theta\widetilde{T}(t)\delta_O(t - \Delta t)\Delta t + \Theta\widetilde{T}(t)(\delta_S(t) + \tau)\Delta t \quad (1)$$

where t is the current model time, Δt is the time step between model times (1 day), δ_O is δ^{15}N of the oyster tissue, δ_S is δ^{15}N of the seston, $\widetilde{T}(t)$ is the normalized temperature as a function of time, Θ is the tissue turnover rate at maximum normalized temperature, τ is the change in δ^{15}N due to a trophic shift. The input seston δ^{15}N values, δ_S, was obtained by interpolating measured seston δ^{15}N values at each simulation time step, t. The normalized temperature values ($\widetilde{T}$) were obtained from water measurements interpolated to model times and normalized to 1 at maximum observed summer temperature and 0 at minimum observed winter temperature. When sampling occurred over only one season (i.e. in Monie Bay), the maximum summer temperature was assumed to be 30 °C and the minimum water temperature to be 0 °C. Finally, the maximum turnover rate (Θ) was determined by minimizing the least squares model error (E) for the seasonal data set from the Choptank River:

$$E = \sqrt{\sum_{i=1}^{n}\left(\delta_O^{\text{measured}}(t_i) - \delta_O^{\text{modeled}}(t_i)\right)^2} \quad (2)$$

where i indexes each of the time steps at which oyster δ^{15}N was measured and σ_i^2 is the standard deviation of that measurement. This error measure is standard to quantify the average deviation between the model and measured δ^{15}N values of the oyster, and is used implicitly in the parameter inference by regression in Moore (2003), Witting et al. (2004), and Sweeting et al. (2005). The resulting parameters for maximum tissue turnover (Θ) were 1.9% day^{-1} in mantle with (E = 4.7‰), 2.6% day^{-1} in gill (E = 3.0‰), and 0.7% day^{-1} in muscle (E = 1.5‰). Literature values report an average trophic shift of 3.4‰ per trophic level (Minagawa and Wada, 1984), so scenarios of 3.0–4.0‰ were used for model verification and error assessment and 3.0‰ was selected as it yielded minimal error. Using these parameters, oyster tissues were modeled and compared to measured data using measured seston δ^{15}N values over time in the Choptank River and further validated against an independent data set from Monie Bay. Finally, a constant value of seston δ^{15}N (8.0‰) within the observed range of seston δ^{15}N values in Monie Bay was used as input to identify the length of time it takes for oyster δ^{15}N to stabilize under a constant maximum summertime temperature (30 °C) and under temperatures observed in the Choptank River across seasons (starting in winter), providing the optimal deployment duration.

3. Results

3.1. Optimal sample size

Because of individual variability in δ^{15}N values in oysters (58 ± 2 mm shell height), it was necessary to determine the number of individual δ^{15}N measurements to average over (the sample size) that best balanced error with effort. For sample sizes of 2–20, the mean, maximum, and minimum standard errors were compared (Fig. 2a–c) and as sample size increased, the maximum and minimum converged upon the mean standard error, becoming increasingly balanced around the mean value, with optimum balance at sample size five. The range of standard errors for δ^{15}N at sample size five for muscle (0.08), gill (0.07), and mantle (0.14) was larger than the minimum range of standard errors for each tissue: muscle (0.03 at sample size 20), gill (0.02 at sample size 17), and mantle (0.02 at sample size 19). However, a minimum of five replicate samples are required (Fig. 2a–c) to optimize standard error.

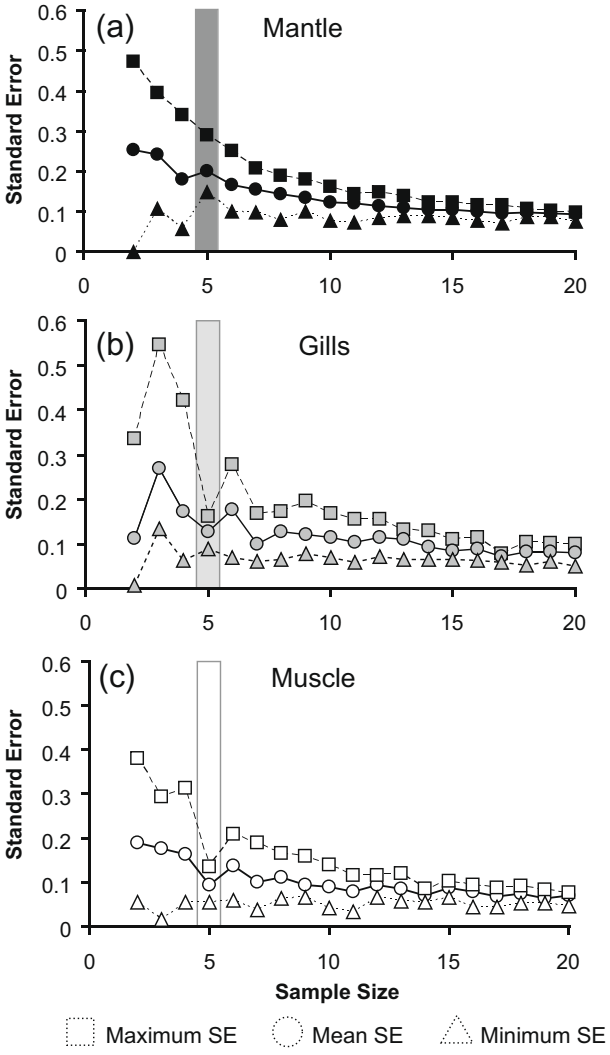

Fig. 2. Trade-off between error and sample size for oyster (a) mantle, (b) gills, and (c) muscle tissues. Panels include the maximum (squares), mean (circles), and minimum (triangles) standard errors of various sample sizes drawn randomly from 35 samples (Bros and Cowell, 1987).

3.2. Convergence and stabilization of δ^{15}N values

Oyster transplantations were conducted in the mesohaline region of Chesapeake Bay, which was characterized by salinity ranging from 0.9 in upper portions of tributaries to 15.0 near tributary mouths and temperatures that ranged from 0.2 °C in winter to 29.1 °C in summer. Oxygen saturation fluctuated from 15.0% to 115.6% and dissolved oxygen concentrations were below 5.0 mg L^{-1} during 32% of measurements in Monie Bay and 61% of those in Choptank River. Secchi depth ranged from 0.3 to 2.8 m, with a mean of 1.0 m.

Initially, oyster δ^{15}N was significantly and consistently higher in oysters from the Severn River (48 ± 2 mm shell height) as compared to the South River (57 ± 1 mm shell height; all tissues $p < 0.01$, Table 1a, Fig. 3a) but after transplantation to Monie Bay, δ^{15}N values in oysters converged to common values at each of the ten stations (Fig. 3a). Oyster tissue δ^{15}N from both rivers declined from initial values (12.3 ± 0.2‰ in South River and 13.7 ± 0.1‰ in Severn River, mean of all tissues; Table 1a) after deployment in Monie Bay (11.3 ± 0.1‰, grand mean of all tissues post-deployment; Table 1b; Fig. 3a) as they fed on seston which also declined in δ^{15}N values during the deployment period

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Table 1
Descriptive statistics and ANOVA analysis of δ^{15}N values and %N in oyster tissues from the South and Severn Rivers (a) and after shared deployments in Monie Bay and its tributary creeks (b). Sample size (n), mean, standard error (SE), degrees of freedom (df), mean square error (MSE), F value (F), and p value (p) are reported.

Parameter	Tissue	South River		Severn River		ANOVA				
		n	Mean (SE)	n	Mean (SE)	n	df	MSE	F	p
(a)										
δ^{15}N (‰)	Muscle	10	12.5 (0.1)	10	14.3 (0.1)	20	1, 18	0.0902	174.86	**<0.0001**
	Gills	5	12.2 (0.2)	5	13.3 (0.1)	10	1, 8	0.1281	23.78	**0.0012**
	Mantle	4	12.2 (0.3)	5	13.4 (0.1)	9	1, 7	0.1966	16.86	**0.0045**
%N	Muscle	10	12.3 (0.2)	10	12.6 (0.2)	20	1, 18	0.5547	1.12	0.3043
	Gills	5	9.7 (0.2)	5	9.8 (0.2)	10	1, 8	0.2011	0.31	0.5958
	Mantle	4	10.1 (0.5)	5	9.8 (0.3)	9	1, 7	0.5790	0.29	0.6046

Parameter	Tissue	Monie Bay		Little Creek		Little Monie Creek		Monie Creek		ANOVA				
		n	Mean (SE)	n	Mean (SE)	n	Mean (SE)	n	Mean (SE)	n	df	MSE	F	p
(b)														
δ^{15}N	Muscle	20	12.9 (0.2)	20	11.9 (0.1)	30	11.9 (0.1)	30	12.4 (0.1)	100	1, 98	0.4843	15.68	**0.0001**
	Gills	20	12.0 (0.2)	20	10.7 (0.1)	30	10.5 (0.1)	30	10.7 (0.1)	100	1, 98	0.6921	2.26	0.1356
	Mantle	20	11.8 (0.2)	20	10.7 (0.1)	30	10.5 (0.1)	30	10.7 (0.1)	100	1, 98	0.5945	1.54	0.2172
%N	Muscle	20	13.9 (0.1)	20	13.1 (0.1)	30	13.1 (0.2)	30	13.5 (0.2)	100	1, 98	0.8760	0.20	0.6539
	Gills	20	9.9 (0.1)	20	9.7 (0.1)	30	9.7 (0.1)	30	10.2 (0.1)	100	1, 98	0.4593	0.12	0.7351
	Mantle	20	9.6 (0.1)	20	9.8 (0.1)	30	9.2 (0.2)	30	9.3 (0.1)	100	1, 98	0.6606	0.26	0.6087

(Fig. 4a and b). Post-deployment, gill and mantle δ^{15}N in oysters from different initial rivers were not significantly different at each of the stations along the δ^{15}N gradient in Monie Bay although muscle δ^{15}N values were still significantly different between sites (Table 1b, Fig. 3a). In contrast to δ^{15}N values, deployment in Monie Bay did not alter oyster nitrogen content (%N), as %N did not vary among initial rivers before or after deployment in Monie Bay (Table 1a and b, Fig. 3b).

Convergence rates of δ^{15}N values varied by tissue and its metabolic activity. When post-deployment δ^{15}N values were plotted to compare oysters from the Severn and South Rivers and exhibited a slope of 1.0, complete convergence of δ^{15}N during deployment was achieved, while plots with slopes <1.0 indicated incomplete convergence. Mantle (fastest tissue metabolism) had a slope of 1.0 (Fig. 5a), gills had an intermediate slope (0.92; Fig. 5b), while mus-

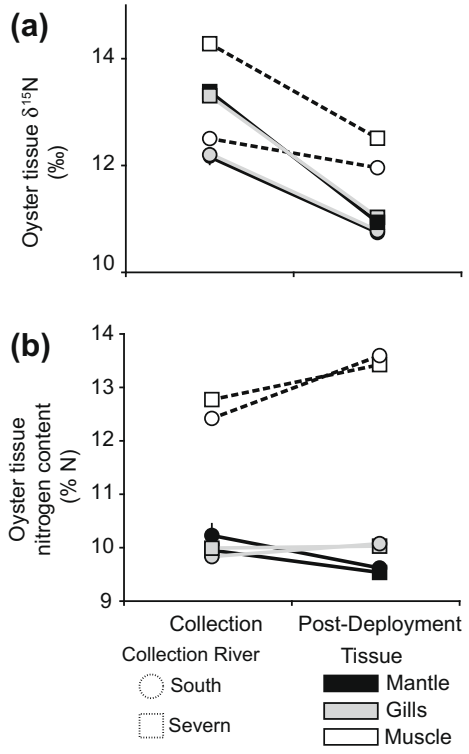

Fig. 3. Mean oyster mantle (black), gills (grey), and muscle (white) δ^{15}N (a) and %N (b) upon collection in the South River (circles) and Severn River (squares) and after deployment in Monie Bay. Standard error bars are plotted, but in most cases are smaller than symbols representing means.

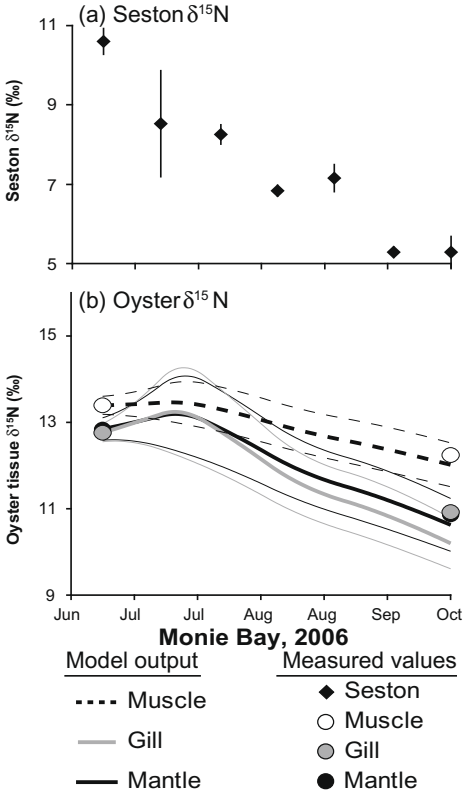

Fig. 4. Measured mean (a) seston δ^{15}N values and (b) modeled and measured oyster δ^{15}N values for mantle (black), gill (grey), and muscle (white) tissues in Monie Bay and its tributary creeks. Standard error bars are presented for measurements, but are sometimes smaller than symbols representing means.

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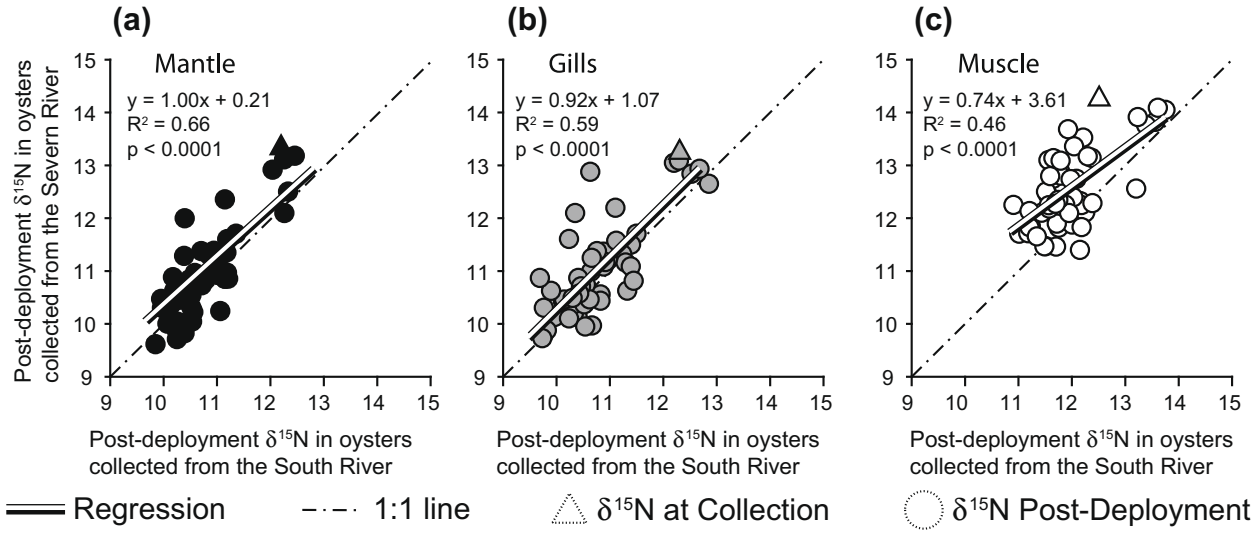

Fig. 5. Comparison of oysters initially grown in the Severn or South Rivers after a shared deployment in Monie Bay. Mantle (a), gills (b), and muscle (c) δ^{15}N values at the initial collection (triangles) and post-deployment (circles) are regressed and plotted against the 1:1 line.

cle (slowest tissue metabolism) had the lowest slope value (0.74; Fig. 5c). Regressions of tissue δ^{15}N from both collection rivers were significant (all tissues $p < 0.0001$; $R^2 > 0.45$), but they did not significantly differ across all tissues ($p > 0.05$). Contrast analyses between slopes indicated that muscle significantly differed from both gill and mantle (df = 1, SS < 4.2, $F = 14.22$, $p < 0.01$) but that mantle and gill (more metabolically active tissues) did not significantly differ (df = 1, SS < 0.10, $F = 0.33$, $p < 0.57$; Proc GLM, SAS Institute).

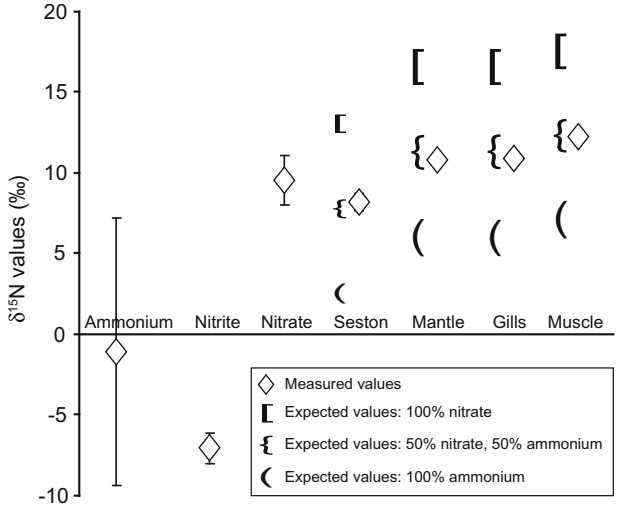

Fig. 6. Mean nitrite, nitrate, ammonium, seston, and oyster tissue (mantle, gills, and muscle) δ^{15}N values. Standard error bars are shown, though in some cases are smaller than data points.

Regardless of completion of convergence in each tissue, the post-deployment oyster δ^{15}N value resembled seston and dissolved inorganic nitrogen δ^{15}N values in Monie Bay. Measured δ^{15}N values in each oyster tissue matched expected values generated from a 50/50 mixture of ammonium and nitrate δ^{15}N values modified by two trophic shifts (Fig. 6). Additionally, measured δ^{15}N values in oyster tissues matched both measured seston δ^{15}N values (Fig. 4a) and modeled oyster δ^{15}N values (Fig. 4b). Therefore, both measurements and modeling indicate that oyster δ^{15}N values resembled ambient signatures in Monie Bay after deployment.

3.3. Required exposure time and temporal integration

Seasonal variations in seston and oyster δ^{15}N values each related differently to water quality metrics. Oysters grew (from a mean of 16 to 61 mm) over the course of the study. Both seston and all oyster tissue δ^{15}N values varied seasonally in the Choptank River (Table 2), and were related to total nitrogen concentrations (Table 3). Seston δ^{15}N values were strongly related to temperature, and were elevated during spring and summer (May–July), depleted in cooler months (November–March; Table 2, Fig. 7a), exhibited a significant positive correlation with temperature and salinity and a significant negative relationship with dissolved oxygen concentrations (Table 3). The relationship between seston δ^{15}N values

Table 2
Descriptive statistics and ANOVA analysis of oyster δ^{15}N values and %N in muscle, gills, and mantle tissues in the Choptank River, 2006–2007. *Spring:* March–May, *Summer:* June–August, *Fall:* September–November, and *Winter:* December–February. Seasons were analyzed by ANOVA. Sample size (n), degrees of freedom (df), mean squared error (MSE), F value (F), and p value (p) are reported.

Parameter	Tissue	Spring		Summer		Fall		Winter		ANOVA				
		n	Mean (SE)	n	Mean (SE)	n	Mean (SE)	n	Mean (SE)	n	df	MSE	F	p
δ^{15}N	Muscle	32	14.3 (0.1)	23	13.8 (0.1)	27	14.4 (0.1)	27	14.2 (0.1)	109	3, 105	0.33	4.41	**0.006**
	Gills	17	14.1 (0.2)	18	13.1 (0.2)	23	14.0 (0.1)	24	14.5 (0.2)	82	3, 78	0.45	17.04	**<0.001**
	Mantle	15	13.9 (0.2)	19	12.7 (0.3)	23	13.6 (0.2)	23	14.3 (0.1)	80	3, 76	0.83	10.37	**<0.001**
%N	Muscle	32	11.9 (0.2)	23	12.0 (0.3)	27	12.2 (0.1)	20	11.2 (0.6)	102	3, 98	2.46	1.64	0.186
	Gills	17	9.9 (0.3)	18	9.5 (0.1)	23	9.3 (0.2)	16	9.8 (0.4)	74	3, 70	1.17	1.19	0.318
	Mantle	15	10.5 (0.6)	19	10.3 (0.5)	23	9.3 (0.2)	14	9.6 (0.3)	71	3, 67	3.07	2.09	0.110

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Table 3
Correlations between physical, chemical, and biological measurements conducted in the Choptank River (2006–2007). Correlations use the lower of the two sample sizes. Significant correlations are in bold and asterisks reflect p values.

	Units	Sample size	Muscle δ^{15}N ‰ 19	Gill δ^{15}N ‰ 15	Mantle δ^{15}N ‰ 15	Seston δ^{15}N ‰ 17	Total phosphorus µM 13	Total nitrogen µM 13	Dissolved oxygen % 18	Dissolved oxygen mg L^{-1} 18	pH 17	Temperature °C 19	Salinity 19
Salinity		19	0.158	−0.029	−0.278	**0.678****	−0.069	**−0.635***	0.272	0.061	−0.262	0.340	1.000
Temperature	°C	19	−0.436	**−0.627***	**−0.589***	**0.704****	**0.612***	**−0.653***	−0.140	**−0.568***	−0.415	1.000	
pH		17	0.283	0.283	0.210	−0.006	−0.174	0.288	−0.015	0.053	1.000		
Dissolved oxygen	mg L^{-1}	18	−0.008	0.532	0.209	**−0.513***	−0.256	0.349	**0.865*****	1.000			
Dissolved oxygen	%	18	−0.181	0.253	−0.150	−0.117	0.264	0.089	1.000				
Total nitrogen	µM	13	**0.569***	**0.672***	**0.684***	**−0.628***	−0.106	1.000					
Total phosphorus	µM	13	−0.216	−0.199	−0.073	0.149	1.000						
Seston δ^{15}N	‰	17	−0.219	−0.385	−0.552	1.000							
Mantle δ^{15}N	‰	15	**0.650****	**0.905*****	1.000								
Gill δ^{15}N	‰	15	**0.725****	1.000									
Muscle δ^{15}N	‰	19	1.000										

* $p < 005$.
** $p < 0.01$.
*** $p < 0.001$.

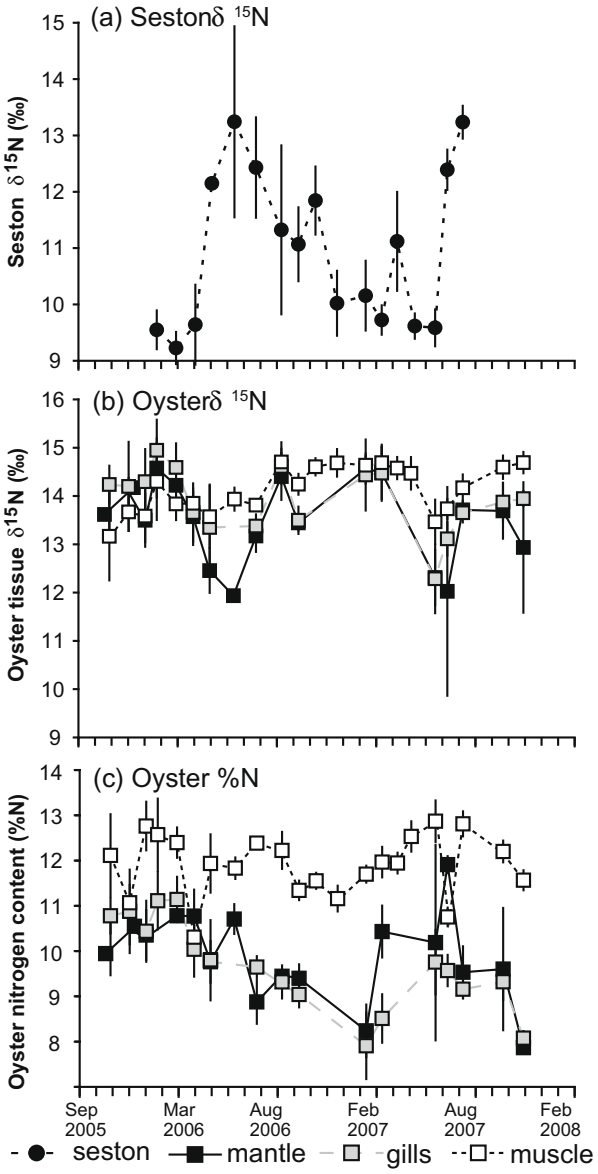

Fig. 7. Seasonal variations observed in the Choptank River of (a) seston (black circles), (b) δ^{15}N values in oyster tissues, and (c) nitrogen content in oyster tissues. Oyster tissues are represented by squares: mantle (black), gills (grey), and muscle (white).

and temperature was significant in both simple linear ($p < 0.03$) and multiple regressions (seston $\delta^{15}N = 7.881 + (0.236$ salinity$) + (0.0847$ temperature$) - (0.0745$ dissolved oxygen mg L^{-1}); mean squared error = 0.795, $R^2 = 0.73$, $p < 0.001$). Converse to seston δ^{15}N, oyster δ^{15}N values tended to be depleted during warmer months (Table 2) and oyster muscle δ^{15}N was not significantly related to any physical metric though mantle and gill δ^{15}N values were negatively correlated to temperature almost as strongly as seston δ^{15}N was positively correlated (Table 3). Mantle tissue δ^{15}N values were most variable between samples while muscle tissue δ^{15}N values were least (Fig. 7b, Tables 1a and b and 2). Despite apparent variations in oyster %N (Fig. 7c), these were not significantly different by season (Table 2). Modeling exercises reflected the variability in oyster tissue δ^{15}N values over time in the Choptank River (Fig. 8a–c). Modeled gill δ^{15}N best resembled measured values (total error of 2.99‰) but had moderate variability, while muscle had minimum variability but moderate total error (1.53‰), followed by mantle, which varied the most and had the highest total model error (4.66‰; Fig. 8a–c).

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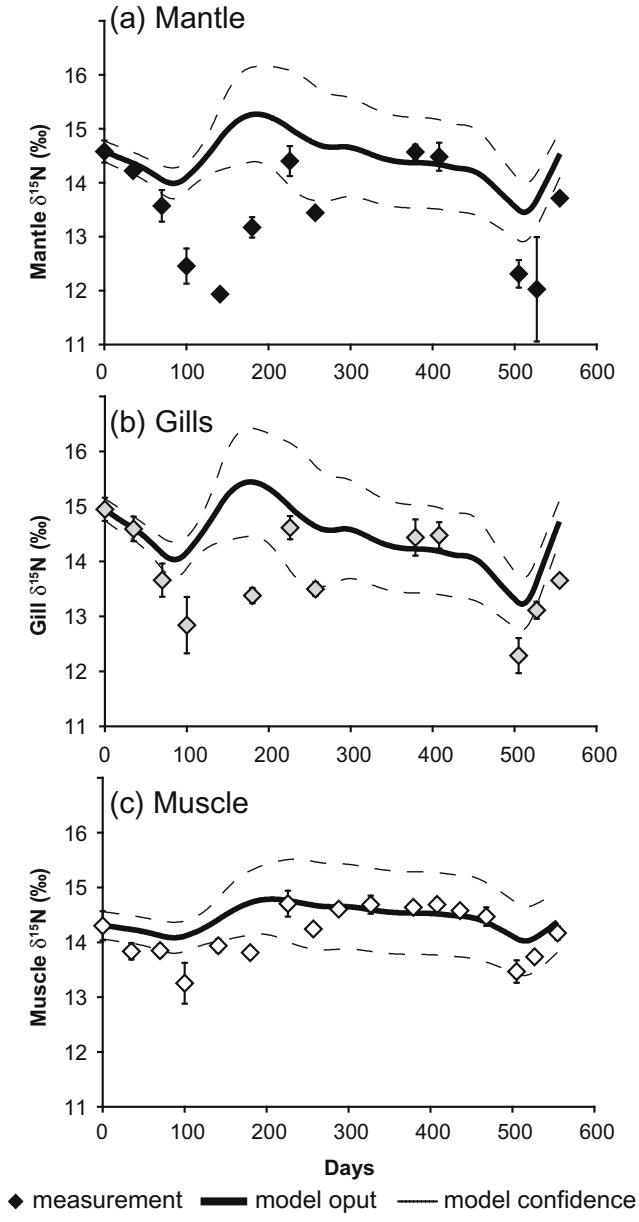

Fig. 8. Modeled and measured values of oyster δ^{15}N values in (a) mantle, (b) gills, and (c) muscle during 2006–2007 in the Choptank River.

Modeled response of oyster δ^{15}N over time in a hypothetical scenario with constant seston δ^{15}N determined the required exposure time to attain stable tissue δ^{15}N. Tissue δ^{15}N values decreased exponentially when a constant summer temperature was assumed and rapidly during warm months and slower during cooler months when seasonal temperatures were used as inputs. Oyster tissue δ^{15}N values stabilized at 11.0 ± 0.5‰, which was offset from the constant seston δ^{15}N input (8‰) by the trophic fractionation factor (+3‰). Under a constant summer temperature, stabilization was achieved quickly (79 days in gills, 103 days in mantle, and 258 days in muscle) compared to seasonal temperature fluctuations: 165 days in gills, 189 days in mantle, and 522 days in muscle (Fig. 9). Maximum turnover rates were reached during summer conditions in this model. Model results indicated longer required exposure times than observations, where oyster tissue δ^{15}N values lagged seston δ^{15}N values by an average of 33–57 days (Table 4), producing the best fit as compared with all other time period combinations during the four month deployment in Monie Bay.

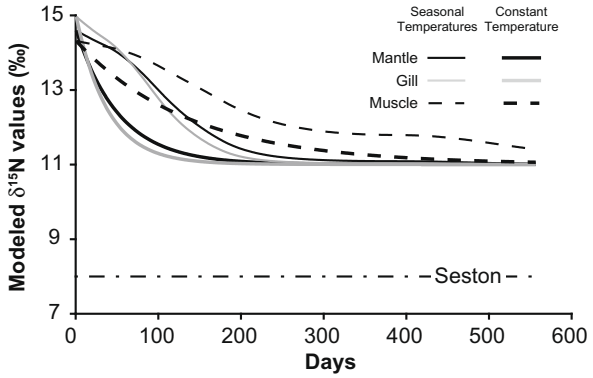

Fig. 9. Hypothetical scenario with a constant seston δ^{15}N value and modeled oyster mantle (black), gill (grey), and muscle (white) δ^{15}N values.

Table 4
Regression analysis between oyster mantle, gill, and muscle δ^{15}N values as the dependent variable and mean seston δ^{15}N values during various time periods as the independent variable. Regression R^2 values are presented for each tissue.

Averaging seston since	Days integrated	δ^{15}N in oysters vs. seston		
		Mantle R^2	Gills R^2	Muscle R^2
22-January	110	0.24	**0.51**	**0.55**
11-July	91	**0.52**	**0.58**	0.20
25-July	77	**0.52**	**0.57**	0.16
14-August	57	**0.65**	**0.69**	0.32
7-September	33	**0.83**	**0.83**	**0.56**
10-October	1	0.14	0.21	0.36

4. Discussion

4.1. Sample size requirements

Quantifying the required replication at the convergence of minimum, mean, and maximum standard errors (Bros and Cowell, 1987) optimized sample size at five individuals (Fig. 2a–c). Relatively low variability (Fig. 2a–c) among individuals required few replicate samples, and is due to temporal integration, but individual variability likely persists due to isotopic heterogeneity in the food supply. Many previous studies (e.g. Costanzo et al., 2001; Elliott and Brush, 2006; Fry and Allen, 2003; McClelland and Valiela, 1998) identified nitrogen source via opportunistic sample collection while others manipulatively examined δ^{15}N in the laboratory or field (Adams and Sterner, 2000; Fertig et al., 2009), but few bioindicator studies explicitly identified appropriate sample size. Small sample size requirements reduced the likelihood of incomplete sampling due to mortality (Ford et al., 2006; Newell et al., 2000), disease (Ford and Smolowitz, 2007), or predation (Kennedy et al., 2009) in addition to enabling future studies at broad spatial scales.

4.2. δ^{15}N converges to reflect deployment locations

Oyster δ^{15}N tissues converge upon a common isotopic signature influenced by ambient isotopic signatures because they are assimilating local nitrogen sources. In Monie Bay, both nitrate and ammonium are available to and assimilated by plankton, with concentrations higher in upstream areas due to anthropogenic terrestrial runoff (Fertig et al., unpublished results). Both seston and all three oyster tissue δ^{15}N values fit expected δ^{15}N values generated from a 50/50 mixture of dissolved inorganic nitrogen δ^{15}N values modified by two trophic shifts (Fig. 6). Additionally, mea-

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sured δ^{15}N values in oyster tissues matched modeled values based upon measured seston δ^{15}N values (Fig. 4a) and measured initial oyster δ^{15}N values (Fig. 4b). Therefore, after deployment, oyster δ^{15}N values resembled the ambient gradient of δ^{15}N signatures in Monie Bay (Fig. 5a–c), and can be averaged to elucidate this convergence (Fig. 3a). Convergence with local ambient conditions enables organisms initially grown in various locations to be utilized, and expands potential areas for deployment even to areas lacking extant or sufficient populations (Smith et al., 2005), as has been done with macroalgae (Costanzo et al., 2001; Udy and Dennison, 1997), filter feeders (Gartner et al., 2002), or a combination (Fertig et al., 2009).

While oyster mantle and gill δ^{15}N values were sensitive to changing conditions, %N as a measure of nutritional condition was not. Tissue δ^{15}N values post-deployment were lower than upon collection (Fig. 3a) while %N did not change substantially (Fig. 3b). Similarly, oyster δ^{15}N values from the Severn and South Rivers initially varied by collection river (Table 1a), but did not significantly differ after shared deployment in Monie Bay (Table 1b). Meanwhile, oyster %N from these two rivers did not significantly differ either before or after deployment (Table 1a and b). As further corroboration, post-deployment δ^{15}N values in oysters from the South and Severn Rivers aligned along the 1:1 line (Fig. 5a–c), from which δ^{15}N responsiveness was inferred. Therefore, δ^{15}N values were sensitive to different nitrogen sources while %N was less responsive, likely because nutritional condition of oysters was relatively stable and because source of dissolved inorganic nitrogen was not directly related to algal production or assimilation by oysters.

4.3. Oyster tissues integrate nitrogen over different time periods

Due to variations by tissue in turnover, nitrogen is integrated over different time periods (e.g. Fry, 2006), with the shortest period in the most metabolically active tissues (mantle, gills) and the longest in less active tissues (muscle). Muscle tissue integrated the longest of the three tissues, as inferred from minimal temporal variability (Figs. 7b and 8c). Further, all tissues from deployments in Monie Bay significantly differed by the initial growth location (Table 1a), but only muscle remained different after deployment (Table 1b). Measurements (Fig. 5a–c), data regressions (Table 4) and modeling exercises (Fig. 9) indicated that muscle tissue converged most slowly, though modeling provided an overestimate (258 days for muscle, 103 days in mantle, and 79 days in gills for a constant summertime temperature) (3–57 days), possibly due to temperatures and growth rates observed in Monie Bay. Longer lengths of time required for the muscle to stabilize likely explains the apparent enrichment (of approximately +1‰) of this tissue compared to gills or mantle (Fig. 9). Similarly, bivalve muscle tissues were less sensitive than viscera (Garton et al., 2005; Moore, 2003; Piola et al., 2006) though likely more sensitive than the shell matrix which integrates over the life of the bivalve (Carmichael et al., 2008). Variations in time integration may result in a longer required deployment time for muscle tissue than mantle or gills, though four months should be sufficient for all tissues and still enables short-term manipulative fieldwork with *C. virginica*, in comparison to species with very slow metabolism, such as the hydrocarbon seep mussel *Bathymodiolus childressi* takes over a year for complete tissue turnover (Dattagupta et al., 2004). Regardless of tissue or species selected, exposure and integration duration should be considered when interpreting nitrogen sources.

Reliance upon on measured temperatures and seston δ^{15}N values enabled the oyster isotope model to estimate seasonal uptake rates and turnover times *in situ* via tissue δ^{15}N value outputs (Fig. 7). Variable environmental conditions and seston δ^{15}N values over seasons extends tissue turnover estimation beyond laboratory conditions (e.g. Witting et al., 2004; Sweeting et al., 2005) or single season field studies (e.g. Moore, 2003) because assumption of a constant input δ^{15}N values is unnecessary. When applied to a scenario with constant temperature and isotopic signature over time (Fig. 9), the oyster isotope model reduces to similar exponential tissue turnover models found in the literature (e.g. Moore, 2003; Witting et al., 2004; Sweeting et al., 2005).

Exposure timing and integration duration influenced seasonal patterns of δ^{15}N values in oysters and their relationship with dietary seston δ^{15}N values. In the literature, few of the numerous studies using δ^{15}N values to indicate nitrogen source account for seasonal variability (Finlay and Kendall, 2007). Summertime enrichment in seston (12.2 ± 0.5‰) was observed in the Choptank River (Fig. 7a) similar to seasonal patterns in Waquoit Bay (York et al., 2007) and in Florida Bay (Anderson and Fourqurean, 2003; Fourqurean et al., 1997, 2005, 2007). As a food source, cyclical patterns of seston δ^{15}N influences oyster δ^{15}N values (Matthews and Mazumder, 2005), which also significantly differed by season (Table 2), but opposite in sign (depleted in summer, enriched in winter) to that of seston δ^{15}N (Fig. 7a and b). As oysters integrated over time and were influenced by previous seasons, they were also more metabolically active in warmer months due in part to temperature-related biochemical reactions; (Dame, 1972). Consistent with assumptions, oyster isotope model outputs exhibited faster responses in warmer months and longer retention of these values into cooler months (Fig. 8a–c) as well as with constant temperature (Fig. 9), implying maximal turnover during summer, the importance of temperature dependence and confirms that spring and summer are ideal times for deployment. Similar to scallops (Lorrain et al., 2002), oyster tissue δ^{15}N values converged in late summer and fall, as metabolite stores are utilized by the gonads during maturation (Fig. 7b). Therefore, oysters were most sensitive when acclimated during spring for summer deployments. Furthermore, mantle was the most responsive tissue to these seasonal shifts (Fig. 7b), reaching lowest δ^{15}N values by summertime (12.7 ± 0.3‰). Muscle δ^{15}N values were also depleted in summer months (13.8 ± 0.1‰), but less so than either gills (13.1 ± 0.2) or mantle (Fig. 7b; Table 2), which suggested this tissue integrated over the longest time period, likely due to slow turnover rates compared to other tissues (Bosley et al., 2002; Frazer et al., 1997; Sakano et al., 2005). Oysters were clear temporal integrators of nitrogen sources as they reflected seasonal δ^{15}N values with less variability than primary producers.

Baseline variations between tissues were possibly due to amino acid composition and associated affinities to δ^{15}N (Gaye-Siessegger et al., 2004), but regardless of mechanism these variations are important to consider when interpreting δ^{15}N values across multiple tissues. Mean muscle δ^{15}N values in all samples except for those in the Choptank River during winter 2005 were greater than mantle or gill δ^{15}N values by 0.4–1.1‰ or 0.2–0.7‰, respectively (Figs. 2a, 6 and 7b). Relatively higher muscle δ^{15}N values compared to other tissues have also been observed in other species including mammals, fish, birds, and bivalves (Frazer et al., 1997; Heikoop et al., 2000; Hobson and Clark, 1992; Lorrain et al., 2002; Piola et al., 2006; Tieszen et al., 1983). If differences between tissue δ^{15}N values were solely due to nitrogen source and integration times, the tissue type that was most enriched would vary more often, especially in the case of a pulse of anthropogenic nitrogen source, however this scenario was not observed.

5. Conclusions

Multiple biological characteristics make oysters appropriate biological indicators of nitrogen sources. Sample size can be opti-

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mized at five individuals, which can be achieved with 7–8 oysters (accounting for mortality) collected from multiple locations for deployment lasting four months (or two to three months, if only analyzing mantle or gill) at new locations to identify nitrogen sources. Manipulative field deployments can provide spatial and temporal integrations (e.g. Costanzo et al., 2001; Fertig et al., 2009). Staggering deployments would enable nitrogen source detection at desired intervals to provide an inference of changes in nitrogen source over weeks, months, or even a year (Dattagupta et al., 2004; Fila et al., 2001; McKinney et al., 2002; Moore, 2003), however due to variations in integration periods and seasonal fluctuations in primary producers, deployment in springtime would optimize integration over summer months. For studies concerned with chronic or long-term nitrogen sources, muscle tissues best provided temporal integration but required a longer exposure time than gills or mantle. Since mantle and gill tissue $\delta^{15}N$ values responded similarly, the mantle would suffice for short-term studies due to slightly quicker response times than gills. This simple and straightforward method may aid detection and monitoring of nitrogen sources over multiple spatial and temporal scales.

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