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From middens to modern estuaries, oyster shells sequester source-specific nitrogen

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Abstract

Oysters (*Crassostrea virginica*) were an important food resource for native peoples of the northern Gulf of Mexico, who deposited waste shells in middens. Nitrogen (N) stable isotopes ($\delta^{15}N$) in bivalve shells have been used as modern proxies for estuarine N sources because they approximate $\delta^{15}N$ in suspended particulate matter. We tested the use of midden shell $\delta^{15}N$ as a proxy for ancient estuarine N sources. We hypothesized that isotopic signatures in ancient shells from coastal Mississippi would differ from modern shells due to increased anthropogenic N sources, such as wastewater, through time. We decalcified shells using an acidification technique previously developed for modern bivalves, but modified to determine $\delta^{15}N$, $\delta^{13}C$, %N, and % organic C of these low-N, high-C specimens. The modified method resulted in the greatest percentage of usable data from midden shells. Our results showed that oyster shell $\delta^{15}N$ did not significantly differ between ancient (500–2100 years old) and modern oysters from the same locations where the sites had undergone relatively little land-use change. $\delta^{15}N$ values in modern shells, however, were positively correlated with water column nitrate concentrations associated with urbanization. When N content and total shell mass were combined, we estimated that middens sequestered 410–39,000 kg of relic N, buried at a rate of up to 5 kg N m^{-2} yr^{-1}. This study provides a relatively simple technique to assess baseline conditions in ecosystems over long time scales by demonstrating that midden shells can be an indicator of pre-historic N source to estuaries and are a potentially significant but previously uncharacterized estuarine N sink.

Keywords: Midden; Acidification; $\delta^{15}N$; Proxy; Archaeology

1. INTRODUCTION

Oysters (*Crassostrea virginica*) were an important food resource for native peoples of the northern Gulf of Mexico (nGOM), who harvested shellfish and deposited waste shells and other artifacts in shell middens (Blitz and Mann, 2000; Jackson, 2015). Oysters have continued to be an important fishery and food source in historical and modern times, with the GOM contributing more than 75% of the commercial oyster landings in the U.S. in

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2013 (National Marine Fisheries Service, 2015). The efficiency of these filter feeders at removing and assimilating nitrogen (N) from suspended particulate matter (SPM) into soft tissue and shell biomass has led to interest in quantifying oysters as long-term N sinks that are not generally considered in ecosystem and N process models for estuaries and coasts (Carmichael et al., 2012b; Kellogg et al., 2014; Dalrymple and Carmichael, 2015). However, little is known about the quantity or chemical characteristics of N retained in bivalve shell among locations through time.

Oysters are potentially powerful biological recorders of environmental organic matter sources, including marine phytoplankton, terrestrial or marsh macrophytes, benthic microalgae, macroalgae, or detritus that contribute to the SPM available as food to estuarine bivalves (Peterson et al., 1985; Peterson and Howarth, 1987). Although oyster shells are mostly $CaCO_3$ (calcite), the shell has an organic matrix (Frémy, 1855; Galtsoff, 1964) with proteins used to help nucleate calcite crystals and for structural support (Nakahara et al., 1980). N and C stable isotope values ($\delta^{15}N$ and $\delta^{13}C$) in organic matrix of modern bivalve shells are correlated with site-specific SPM $\delta^{15}N$ and $\delta^{13}C$ values (Geist et al., 2005; Carmichael et al., 2008; Kovacs et al., 2010), which in turn are correlated with N and C sources to estuaries. These correlations indicate that bivalve shell organic matter derives from the bivalve's food and that isotope values in shells may be used as a proxy for soft tissue and SPM (with metabolic offsets; Carmichael et al., 2008, 2012a; Kovacs et al., 2010). As shell is sequentially deposited and preserved, it is more representative of dietary information from a bivalve's entire lifetime than soft tissue (O'Donnell et al., 2003), and it is preserved on much longer timescales than soft tissue.

Ancient bivalve shells, such as preserved in middens or reef outcrops, have similar potential to preserve valuable information about centuries-old organic matter sources. O'Donnell et al. (2003) speculated that $\delta^{15}N$ and $\delta^{13}C$ values in *Mercenaria mercenaria* shell demonstrated shifting food sources between ancient and modern clams, but did not consider changes in land-derived N sources across the timescale of study (Carmichael et al., 2008). More recently, N stable isotope values in modern bivalve shells have been correlated with site-specific N loading rates, wastewater N sources (Carmichael et al., 2008; Kovacs et al., 2010), and patterns of urbanization and industrialization (Versteegh et al., 2011). Few studies, however, have tested these relationships in ancient oyster shells (Andrus, 2011). Black (2014) and Oczkowski et al., (2016) used midden and museum collections of *M. mercenaria* and *C. virginica* shells to begin to define the proxy potential of these ancient shells, determining that ancient and modern shells had different $\delta^{15}N$ values. Calibration of methods and the relationships between ancient and modern shells relative to a range of natural and anthropogenic N sources are needed for broader application of this potential proxy. For example, in many estuarine systems unaltered by anthropogenic N, SPM has lower $\delta^{15}N$ values upstream than downstream (Kendall et al., 2007), suggesting that bivalves growing there will reflect relative influence of freshwater compared to marine-derived N. Therefore, bivalve shell $\delta^{15}N$ has potential use as an indicator of relative influence of freshwater compared to marine sources of SPM in unaltered ancient and modern estuaries.

Existing methods for stable isotope determination in modern bivalve shells have not been optimized for ancient shells. Methodological challenges that must be addressed (Table 1) include, in particular, determining $\delta^{15}N$ values in a very low-N, high-C substrate, and the effects of acidification methods on $\delta^{15}N$ results. Ideally, samples would be manipulated as little as possible before stable isotope analysis (Jacob et al., 2005; Mateo et al., 2008), but even for modern shells, analyzing bulk (not decalcified by acidification) samples can result in low-N samples that can be challenging to analyze (Carmichael et al., 2008; Schlacher and Connolly, 2014). Without appropriate instrument accommodation, large sample sizes and high C concentrations can lead to incomplete combustion or formation of carbon monoxide that overloads detectors, affects instrument timing, and could shift measured ^{15}N values within a sample run (Werner and Brand, 2001). Versteegh et al. (2011) successfully measured $\delta^{15}N$ values in unacidified *Mytilus edulis* shells using a CO_2 trap, but in their methodological analysis of combinations of synthetic $CaCO_3$ and acetanilide, all samples had relatively high %N (>1.6% N) compared to oyster shells (0.16–0.46%N, Higgins et al., 2011; Kellogg et al., 2014; Dalrymple and Carmichael, 2015), and it is unknown whether this method would be successful for ancient shells that may retain even less N (Hudson, 1967). Carmichael et al. (2008) and Kovacs et al. (2010) successfully measured $\delta^{15}N$ values in low-N shells of modern bivalves by decalcifying shells with dilute (1 N) HCl, then filtering the residual organic material onto glass fiber filters. Although this method concentrates organic matter for analyses, it does not quantify organic matter loss during filtration, and neither bulk analyses nor existing acidification methods allows simultaneous determination of $\delta^{13}C$, which can give additional information on food sources to bivalves and be diagnostic of complete acidification and/or combustion of samples during analysis.

Diagenetic changes to $\delta^{15}N$ and $\delta^{13}C$ values in shell organic matter through time are also of concern when analyzing ancient samples. Little study has been done on diagenesis, the chemical or physical alteration of materials, in archaeological shells after burial in sediments. Potential indicators of shell diagenesis include fractionation of amino acids (Macko and Estep, 1984; Qian et al., 1992) and shifting stable isotope values and C:N in the organic matrix of shells (O'Donnell et al., 2003). It is unknown whether diagenesis actually alters bulk $\delta^{15}N$ values in ancient oyster shell, but determining whether diagenesis has occurred in ancient samples on a scale large enough to change interpretation of results is important for accurate determination of the stable isotope values in shells. In general, the relationship between diagenesis and N isotope values in biogenic carbonates is not well understood, with studies on fossil bone collagen (Nelson et al., 1986; Tuross et al., 1988; Ambrose, 1990) and bird shell (Johnson et al., 1998) showing equivocal effects of diagenesis on bulk $\delta^{15}N$. Quantifying decomposition rates or changes in %N of ancient shells can give important information on diagenesis, and will also

Table 1
Summary of potential benefits and drawbacks to methods used for $\delta^{15}N$ in bivalve shell. Methods include bulk (direct combustion), A-F (acidification-filtration), A-C (acidification-centrifugation), and A-O (acidification-other or not specified).

	Method				Citation
	Bulk	A-F	A-C	A-O	
Method Benefits					
CO_2 trap allows analysis of higher C conc. without acidification	x				Versteegh et al. (2011), Black, 2014, Present study
Less sample manipulation	x				Present study
Negligible N loss	x				Present study
$\delta^{15}N$ within 80% of $\delta^{15}N$max when samples >70 μg N		x			Carmichael et al. (2008)
Concurrent analysis of organic $\delta^{13}C$: diagnostic		x	x		O'Donnell et al. (2003), Present study
No significant difference in $\delta^{15}N$ for shell or whole bivalves	x	x	x	x	Mateo et al. (2008), Present study
Allows concentration of shell organic matter in low N samples		x	x		Carmichael et al. (2008), Present study
Method Drawbacks					
Too little N for accurate $\delta^{15}N$ in some samples	x				Black (2014), Present study
Incomplete burning of C, tailing of C in some samples	x				Present study
Incomplete burning of C, CO formation in some samples	x				Present study
Use of CO_2 trap: no simultaneous $\delta^{13}C$ measurements	x				Black (2014), Present study
Avg $\delta^{15}N$ for acidified −0.15 times non-acidified samples (no bivalves)				x	Jacob et al. (2005)
Serial additions of dilute acid decrease reaction efficiency		x			Delong and Thorp (2009), Present study
Soluble matrix, different proteins than insoluble fraction		x	x		Crenshaw (1980), Risk et al. (1996)
Incomplete acidification can lead to inaccurate δ13C		x			Delong and Thorp (2009), Present study
Non-quantifiable N or significant N loss		x	x		Carmichael et al. (2008), Present study
More time-consuming than bulk analysis		x	x		Versteegh et al. (2011)

allow estimates of how long N may be sequestered in middens or relict oyster reefs. Understanding of how oyster shell %N and $\delta^{15}N$ values vary with environmental conditions and through time will aid in our understanding of how oysters contribute to N sequestration and subsequent cycling in estuarine and coastal systems.

The objectives of this study were to (1) test the use of $\delta^{15}N$ values in shells of oysters from archeological sites as a proxy for ancient (pre-colonial) N sources, (2) improve analytical methods for determination of stable isotope and organic matter content in shell, and (3) use shell %N to estimate relic N in middens and quantify middens as a source for long-term N sequestration. To accomplish these goals, it was necessary to optimize $\delta^{15}N$ analytical methods to allow comparison between ancient and modern (transplanted and wild harvested) oyster shells among different sites in the northern Gulf of Mexico. To test effectiveness of oyster shells as an environmental proxy for N source, we compared $\delta^{15}N$ values in modern oyster shells to soft tissues, SPM, and nutrient concentrations across a land-use gradient. We expected $\delta^{15}N$ values in shell-derived organic matter from midden oysters and those at sites with little land-use change to be relatively low, reflecting typical estuarine food sources to oysters (phytoplankton and some marsh- or terrestrially-derived detritus) and low anthropogenic wastewater input compared to oyster shells from sites with major anthropogenic inputs.

To determine the most effective analytical method to reliably measure stable isotope values in ancient shells, we compared results among three sample types; bulk shell, acidified-filtered samples (Kovacs et al., 2010), and acidified-centrifuged samples (a modified acidification method). We hypothesized that our modified analytical method would reliably determine $\delta^{15}N$ values and organic matter content in ancient oyster shell, the resulting stable isotope values in modern and ancient midden shells would vary relative to temporal and spatial differences in land-use change and N source shifts, and %N values would allow us to quantify relic N retained in middens through time. These methods allow midden oyster shell to be used as a proxy to better understand ancient sources of N to marine systems, trace changes in N source through time, and determine if middens may serve as a long-term N sink.

2. MATERIAL AND METHODS

2.1. Ancient middens

In the area now designated as the Grand Bay National Estuarine Research Reserve (GBNERR) in Mississippi, human occupation has been dated as early as 800 BCE (Jackson, 2015), but earlier occupations may lie below modern sea level. We excavated shells from three middens in 2010 (Fig. 1), including: (1) 22JA564 (Bayou Heron), an intact midden (surface area ~1,800 m^2) where shells accumulated slowly through time (on average 0.04–0.07 cm y^{-1}), (2) 22JA575 (North Rigolets), a sparsely vegetated shell heap (surface area ~12,500 m^2) characterized by storm-reworked shell in upper layers and sudden large deposition events throughout (on average 0.50 cm y^{-1}), and (3) 22JA633 (Bayou Cumbest), an intact midden (surface area ~9,000 m^2) that had a combination of fast and

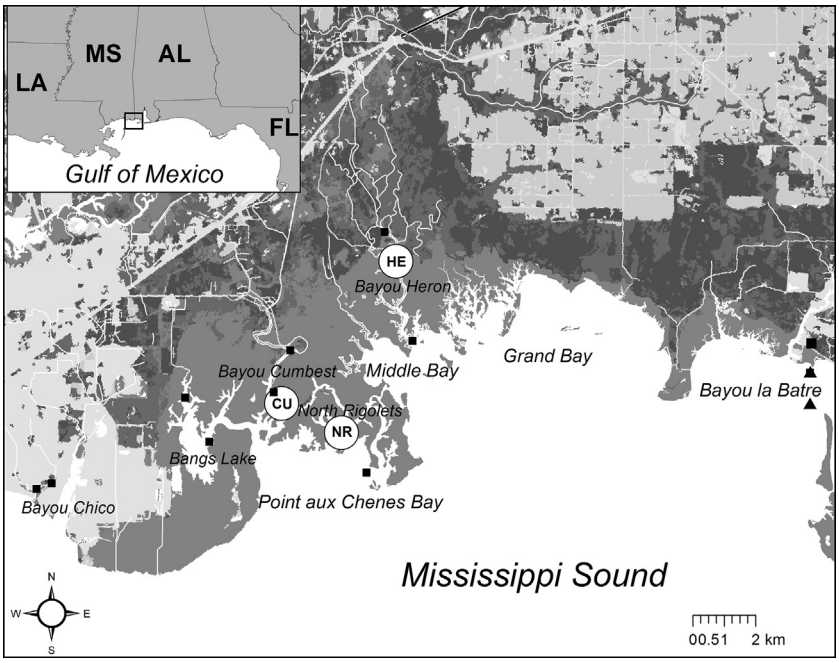

Fig. 1. Map of study area, including locations of middens (Bayou Cumbest (CU): 22JA633; North Rigolets (NR): 22JA575; Bayou Heron (HE): 22JA564), and locations for collection of modern wild shells or deployments of transplant oysters (Bayou Chico, Bangs Lake, Bayou Cumbest, North Rigolets, Bayou Heron, and Bayou la Batre). Black squares are locations of suspended particulate matter (SPM) samples. Shading of land indicates modern land use: pale gray is developed land (high impervious surface), medium gray is marsh, and dark gray is wooded in present day (U.S. Geological Survey, 2009).

slow shell accumulation periods (Jackson, 2015, average 0.46 cm y^{-1}). The midden sites are located in watersheds now dominated by marsh and pine woodlands that have low human population density, and land use has changed little through time compared to surrounding areas along the nGOM coast (Fig. 2).

All shells for this study were retrieved from 1×1 m archaeological units, which were excavated in 10–20 cm levels to the depth of the water table or the limit of cultural deposits, up to 100 cm depth (Table 2). Within each archaeological unit, all material was sieved through 0.64 cm mesh by level and sorted into categories of fragmentary (shell hash, broken shell) and whole shell. Fragmentary shells were measured by volume and mass. Whole shells were counted and shell height was measured in the field (nearest mm). Left oyster valves were sampled for stable isotopes from 2 or more levels within each midden.

Cultural artifacts from associated horizons were used to estimate chronological eras (Jackson, 2015), and six shells were radiocarbon (^{14}C) dated using AMS spectroscopy by Beta Analytic, LLC following Talma and Vogel (1993) and Reimer (2013), calibrated using the MARINE13 database to convert conventional ^{14}C ages to calendar years (Table 2). To determine the accuracy of the MARINE13 reservoir effect correction (Simms et al., 2007), we compared the calibrated and corrected ^{14}C date from one oyster shell to a charcoal (wood) sample from the same level (Jackson, 2015). This sample's date was within one standard deviation of the calibrated calendar age, so we used ages from both charcoal and shells using the MARINE13 correction. Shells of minimal wear were sampled to maximize organic matter integrity and represent the range of ancient time periods represented by the middens (Table 2). Ancient shells were too fragile to endure abrasive cleaning, and were gently rinsed and soft brushed to remove as much

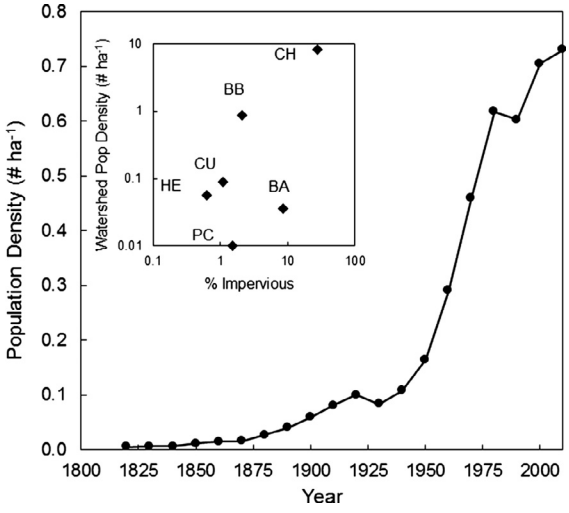

Fig. 2. Population density of Jackson County, MS through time (U.S. Census 2010), showing major population expansions in the late 18th century and mid-20th century. Inset (derived from Darrow et al., 2016) shows relative present-day watershed population density for each compared to % impervious land cover. Sites include Bayou Heron (HE), Point aux Chenes Bay (PC, close to NR midden site), Bayou Cumbest (CU), Bangs Lake (BA), Bayou la Batre (BB), and Bayou Chico (CH). BB and CH have known wastewater inputs.

sediment as possible without affecting the integrity of the shell.

2.2. Modern oysters

To define site-specific relationships between modern shell, soft tissue, and local organic N and C sources (oyster food), we sampled wild and transplanted oysters near midden sites. Wild populations were hand-collected twice from subtidal patch reefs in Bayou Heron, Bayou Cumbest, North Rigolets, Bangs Lake, and Bayou Chico (Fig. 1) ($n = 30$ tissue samples, 15 shells) in October 2012 and May 2013. We also deployed transplanted oysters to examine a larger set of sites with a range of land uses, where we could not find modern wild populations. These sites included adjacent watersheds with higher population density, impervious surface area, and known wastewater inputs (*i.e.,* Bayou Chico, a tidal creek in the city of Pascagoula, and Bayou la Batre, a coastal site near a wastewater treatment plant outfall, Figs. 1 and 2). Transplanted oysters were native aquaculture stock (Auburn University Shellfish Lab) grown subtidally in 0.25×0.25 m wire mesh cages 0.25–0.5 m above sediment surface at 9 sites in the nGOM, including Bayou Heron, Bayou Cumbest, Point aux Chenes Bay, Bangs Lake, Bayou Chico, and Bayou la Batre (Fig. 1). Oysters were transplanted during June–September 2011 (summer season only) and June 2012-May 2013 (year-round). Transplanted oysters that had come to equilibrium with the environment (*in situ* 3 months or longer, $n = 47$ individuals) were used for stable isotope analyses.

Oysters were stored frozen (−20 °C) until processing, when shells were cleaned of sediment and epibionts, and soft tissues dissected from shell. Adductor muscle only was used for stable isotope analysis because it is a longer-term integrator of diet than other tissue types, not contaminated by gut contents, and less isotopically variable than other tissues (Paulet et al., 2006; Piola et al., 2006; Yokoyama et al., 2008). Soft tissues were gently rinsed with ultrapure water, dried at 60 °C, ground to a powder using a mortar and pestle, and stored dry until stable isotope analysis.

2.3. Shell processing

Ancient and modern shells were cut laterally from hinge to outer margin using an Isomet low-speed saw with diamond blade. Shells were rinsed with ultrapure water and thoroughly air-dried for 7–10 days. Shells were ground to a fine powder in a Misonix workstation (FE 2620) using a Ryobi Dremel tool (no. 81-84-9548) with ruby sanding attachment and collected into an acid-washed 20 cm glass dish. We sampled the lateral cut surface of each entire shell half, grinding away and discarding 1 mm external portions of the shell including foreign organic matter, periostracum (when present), and any bore holes. This cleaning technique was found to be most suitable for our sample type to avoid contamination and preserve sample integrity (Fig. S1).

To remove carbonates and extract the acid-insoluble shell organic matrix for stable isotope analysis, we used two methods: (1) an established filtration method ("filter"; Carmichael et al., 2008; Kovacs et al., 2010), and (2) a modified centrifugation method ("centrifuge") that replaced the filtration step in the established method with centrifugation. Briefly, for the filter method, 250 mg (modern) or 500 mg (midden) shell powder from individual oysters was acidified in 20 ml acid-washed glass vials using 5–8 ml 0.5% $PtCl_2$ in 1 N HCl solution, then gently filtered on pre-weighed, combusted (450 °C, 30 min) glass fiber filters (0.7 μm nominal pore size). Filters were minimally rinsed with ultrapure water and dried at 60 °C for 24 h. To estimate % recovery of shell N for comparison to the centrifuge method, we took care to avoid sample loss at each step of the method by rinsing all particles from the filtering apparatus, re-weighing vials to account for sample loss, and noting where material was lost during processing.

For the centrifuge method, 250 mg of ground shell powder was weighed into individual acid-washed, pre-weighed 15 ml polypropylene tubes. 1 ml HCl-$PtCl_2$ was added to the tube and vortexed to thoroughly mix. A second 1 ml of HCl-$PtCl_2$ was added after 5 min, vortexed again, and repeated until no further reaction (bubbling) occurred for a total 3 ml of HCl-$PtCl_2$ and 3 times vortexing of the sample. Samples were centrifuged on low speed (1800 G for 5 min) to concentrate organic matter in the bottom of the tube. The supernatant was decanted, and the HCl-$PtCl_2$ extraction was repeated as described above and allowed to react for 1 h before centrifuging a second time. Additional reaction time was allowed if a solid pellet did not form during centrifugation (dark gray-brown colored, with no white flecks of carbonate) or supernatant was not clear. To avoid acid residue in dried samples confounding sample weight, samples were rinsed with 9 ml of ultrapure water, tubes were vortexed, and samples were centrifuged a final time. Water was decanted, and samples were dried in the tubes at 60 °C for 24 h. After drying, tubes with sample were weighed to quantify recovery. Dried oyster shell organic matter from the centrifuge method was homogenized within the tube by crushing with a clean metal spatula.

To test the need for an acidification procedure to concentrate organic matter before analysis under currently available commercial IRMS instrumentation and control for effects of sample handling, a subset of midden and modern wild oyster shells were analyzed unacidified (bulk samples). For these samples, ground shell powder was directly used for stable isotope analysis without any additional processing or handling ("bulk") samples. For bulk oyster shell powder, less total shell powder was used than acidification methods (17.0 ± 0.2 mg for modern wild oysters, 50.0 ± 0.5 mg for ancient oysters). We analyzed the full range of recovered quantities from acidification methods (0.9–106.0 mg for filtered samples, 0.6–16.7 mg for centrifuged samples).

2.4. Modern organic matter source sampling

When modern oysters were collected, we also collected site water to determine stable isotope values in suspended particulate matter (SPM) and concentrations of dissolved nutrients (Fig. 1). We used a Wildco horizontal seston sam-

Table 2
Characteristics of midden sites (see Fig. 1) and depths from Jackson (2015) from which ancient shells were excavated, and stable isotope range of shells from each site. If shell stable isotope data did not pass quality assurance/quality control criteria (QA/QC), they were not included in further analyses.

Site	Provenience	Depth (cm)	Catalog #	*n*	Comments	Est. age (y)[1]	^{14}C age (y)[2]	$\delta^{13}C_{shell}$ (‰)	$\delta^{15}N_{shell}$ (‰)	$\%N_{shell}$	Passed QA/QC
CU (22JA 633)	N491 E563	0–10	248	4	One of the deepest areas of		550	−22.0 to −21.3	4.9–10.7	0.025–0.037	Y
		10–20	249	1	shell deposit on site	1010		−22.3	5.6		Y
		20–30	250	4			820	−23.8 to −21.3	5.8–8.5	0.020–0.040	Y
	N491 E550	43–50	219	3	Roasting facility feature above this level (20–30 cm)		490[3]	−23.4 to −23.7	11.6–15.0		N
	N492 E550	40–50	133	4	Adjacent to N491E550 but further from shore and fewer shells		2420	−24.4 to −23.5	7.1–11.0		Y
HE (22JA 564)	N490 E496	20–40	24	2	Shovel testing pit	1510		−23.6	5.0		Y
		40–60	25	3			1270	−24.9 to −21.3	2.9–6.9		Y
		60–75	26	1		2009		-22.6	6.1		Y
	N491 E494	10–20	131	1	Compact shell deposit in	1310		−25.0 to −23.9	5.8–6.5	0.030	N
		20–30	135	5	organic matrix; historic		1340	−24.5 to −23.0	6.1–6.9	0.018–0.024	Y
		50–60	85	2	campfire feature above shell (10–20 cm depth)	2009		−24.0	6.4–6.7		Y
NR (22JA575)	N499 E487.11	0–25	10	3	Shovel testing pit	1010		−23.2 to −22.0	6.9–7.1	0.05–0.11	Y
		66–86	16	1		1010		−21.9	7.4		Y
		100–120	18	5		1010		−22.5 to −21.5[4]	5.9–6.6[4]	0.039–0.065	Y
	N505 E506	30–40	77	1	Primarily whole shell in an earth and crushed shell matrix; deposit goes deeper than water table at 80 cm		760	−23.1	4.8	0.026	N
	N494.7 E470.7	40–50	103	1	Shovel testing pit	1310		−19.6[4]	1.7–5.5	0.017	Y

[1] Estimated age based on approximate earliest dates of associated cultural artifacts (Jackson, 2012).
[2] Earliest age based on ^{14}C dates of shell, corrected for marine reservoir effects using MARINE13 database.
[3] Earliest age from range of ^{14}C dates of charcoal from deposit (Jackson 2012).

pler to take 2x1L samples from the depth of oyster transplant cages. Water samples were pre-screened through 200 μm mesh to remove large non-food particulates and returned on ice to the lab, where they were filtered (pre-combusted glass fiber filter, 0.7 μm nominal pore size). Filters were dried at 60 °C for 24 h and stored dry in a desiccator until they were packed for stable isotope analysis. To quantify dissolved nutrients, we measured NO_3^-, NO_2^- and NH_4^+ (dissolved inorganic nitrogen: DIN) in filtrate using a Skalar autoanalyzer with colorimetric methods after cadmium reduction to NO_2^- and using a standard alkaline phenol method (Strickland and Parsons, 1972). Total dissolved nitrogen (TDN) was quantified using the persulfate oxidation method (Bronk et al., 2000), and dissolved organic nitrogen (DON) was calculated by subtracting DIN from TDN.

2.5. Stable isotope analysis

All samples were packed in tin capsules and analyzed at the UC Davis Stable Isotope Facility for $\delta^{13}C$ and $\delta^{15}N$ using a PDZ Europa 20–20 isotope ratio mass spectrometer (IRMS). Both elements were analyzed to determine if the modified acidification method allowed dual isotope determination and if the resulting $\delta^{13}C$ values could be used to assess complete acidification and/or combustion of samples during analysis and to better characterize food sources to bivalves. Samples on filters and bulk shell were combusted by an Elementar Vario Micro Cube elemental analyzer and centrifuge samples were combusted by PDZ Europa ANCA-GSL elemental analyzer, before IRMS.

To determine effects of acidification on standards and oyster organic matter, blank filters, acetanilide standards, and oyster adductor muscle from corresponding shells were similarly processed and analyzed alongside each set of shell samples. Facility internal standards included bovine liver, nylon 5, and glutamic acid. Accuracy of mean $\delta^{15}N$ and $\delta^{13}C$ values of standards with respect to published values was ±0.2‰ and ±0.3‰, respectively. Precision of standards varied by sample type and instrument. For acidified and centrifuged samples, precision (standard deviation, SD) of standards was ±0.08‰ for $\delta^{15}N$ and ±0.23‰ for $\delta^{13}C$. For acidified and filtered samples, standard precision was ±0.11‰ for $\delta^{15}N$ and ±0.47‰ for $\delta^{13}C$. For bulk samples, standard precision was ±0.37‰ for $\delta^{15}N$. The lower analytical limit for these IRMS systems was 20 μg N. %N from blank filters and tins was negligible, and $\delta^{15}N$ and $\delta^{13}C$ values of blanks were below analytical limits of the instruments.

2.6. Data analysis

2.6.1. Analytical precision

Stable isotope data were analyzed for quality and precision before conducting statistical tests following a number of quality assurance-quality control (QA/QC) procedures. $\delta^{15}N$ values for samples <20 μg N were omitted if replicate values had a difference of >0.2‰. We analyzed distributions of $\delta^{15}N$, $\delta^{13}C$, and organic C:N values (all reported C:N values are for organic matter) by shell type and examined outliers for indications of low %N and poor reproducibility due to low sample mass as well as excess carbonate or incomplete acidification (indicated by high $\delta^{13}C$ with high C:N), potential contamination with other substances (e.g.; wood smoke from roasting pits; Black, 2014), or diagenetic alteration. If outliers showed evidence of two or more of these indicators, samples were omitted from further analyses. For shell type, site, and processing method comparisons, only shells from sites Bayou Cumbest, North Rigolets, and Bayou Heron were used because these were sites with both ancient and modern wild shells ($n = 55$ shells). We used data from all sites (Bayou Chico, Bangs Lake, Bayou Cumbest, Bayou Heron, North Rigolets/Point aux Chenes Bay, Bayou la Batre) for analyses of the effects of land-use change on modern shell isotope values in wild and transplanted oysters.

2.6.2. Shell treatment methods

To examine differences in shell processing methods, means for $\delta^{15}N$ and %N were compared using two-way ANOVAs for method (3 levels: filter, centrifuge, bulk), shell type (2 levels: midden, modern wild) and the interaction between method and shell type. The bulk method did not allow analysis of organic C; thus, $\delta^{13}C$, %C, and organic C:N could not be determined, and two-way ANOVAs for these response variables had two levels for method (filter, centrifuge). Whole shell %N was calculated by scaling up %N in IRMS samples (organic fraction for acidified samples) to the mass of shell powder originally analyzed (Table 3). We calculated method yield by comparing %N between bulk and two acidification methods. We quantified error in the %N estimates based on analytical limits and estimates of sample recovery during processing by recording the number of samples having poor reproducibility due to low N after acidification and sample recovery. We excluded these data from subsequent analyses for shell type comparisons. Where there were no significant differences in results among shell processing methods, we combined data for subsequent statistical tests.

2.6.3. Comparison between ancient and modern shell

To examine differences between ancient and modern shells, we tested the effects of site and shell type on $\delta^{15}N$, %N, $\delta^{13}C$, $\%C_{org}$, and organic C:N values using two-way ANOVAs for site (3 levels: Cumbest, North Rigolets, Heron), shell type (2 levels: ancient and modern wild, or modern wild and modern transplant), and interactions between site and shell type. Post-hoc multiple comparisons were conducted using Tukey's HSD for significant ANOVA results. We used linear regression to determine the relationship between response variables and shell age, determined by ^{14}C dating. Data for all analyses were tested for normality and ln-transformed when needed to achieve homogeneity of variance, with all statistical tests conducted using JMP Pro v.11 and a significance level of $\alpha = 0.05$.

2.6.4. Estimates of relic N in middens

Scaled-up estimates of midden N content were made using densities of whole shell, broken shell, and shell hash for each vertical level in a $1 \times 1\ m^2$ unit given by Jackson

Table 3
Comparison of shell treatment methods for stable isotope and N and C content analyses. Sample mass analyzed is the amount of shell material post-processing that was packed for EA-IRMS analysis. Higher mass for filter samples may include residual carbonate due to incomplete acidification. Percentage of samples quantifiable for %N was estimated based on numbers of samples with shell material loss during processing and numbers of samples eliminated due to poor replication or low N quantity. Minimum N mass recommended for $\delta^{15}N$ analytical precision on these IRMS systems was 20 μg N, but some samples <20 μg N were used for stable isotope analysis based on sample replication within 0.2.

Shell type	Method	Total number of samples	Shell powder mass acidified (mg)	Sample mass analyzed (mg)	% of samples quantifiable for %N	Mean N mass for IRMS (μg)	% of samples >20 μg N	% of samples used for $\delta^{15}N$
Ancient	Filter	4	250	41.2 ± 14.5	75	18 ± 9	50	50
	Centrifuge	9	250	5.45 ± 1.35	86	17 ± 3	33	78
	Filter	39	500	84.6 ± 9.7	71	49 ± 6	74	72
	Bulk	17	–	49.8 ± 0.09	100	16 ± 1	30	88
Modern/Wild	Filter	15	250	41.3 ± 2.7	80	267 ± 28	100	100
	Centrifuge	12	250	4.83 ± 1.24	100	200 ± 30	100	100
	Bulk	13	–	16.98 ± 0.01	100	24 ± 4	46	92
Modern/ Transplant	Filter	24	250	36.0 ± 2.8	33	401 ± 38	100	75
	Centrifuge	23	250	6.10 ± 0.93	100	317 ± 30	100	65

(2015), then multiplying by mean shell mass per level and shell %N (detailed calculations in Supplemental Material). Integrated shell N mass was scaled up to a midden by averaging shell N mass per unit and multiplying by the volume of the midden approximated using surveys and shovel tests by Jackson (2015). Low- and high-end estimates were calculated using the range of values for %N and shell mass from each unit. Estimates of shell N burial rates were made by first estimating shell vertical accumulation rate ($m\,y^{-1}$) from the difference in ^{14}C dates established for shells or charcoal excavated from multiple levels within a unit and dividing by the vertical distance between these dated artifacts. N burial rates were determined by multiplying shell accumulation rate by midden shell-derived N content for each level, then averaging for each midden.

3. RESULTS

3.1. Effects of shell type and site

There was no significant difference in $\delta^{15}N$ values between ancient and modern wild oyster shells ($F_{1,46} = 0.42$, $p = 0.52$, Fig. 3a). Shell $\delta^{15}N$ values were higher at site Cumbest than Heron for both ancient and modern wild oysters ($F_{2,46} = 6.64$, $p < 0.01$, Tukey's <0.05, Fig. 3a, Table 4). $\delta^{15}N$ values in modern wild and modern transplant shells were not significantly different due to an interaction between shell type and site: transplant shells from North Rigolets had higher $\delta^{15}N$ values than wild shells from Heron, with other combinations of sites and shell types having intermediate $\delta^{15}N$ values (interaction $F_{2,23} = 26.3$, $p < 0.01$, Fig. 3c). $\delta^{13}C$ values in shells also did not differ between ancient and modern wild samples ($F_{1,42} = 0.003$, $p = 0.96$), but differed by site ($F_{2,42} = 6.18$, $p < 0.01$), with pairwise comparisons showing that North Rigolets > Cumbest and Heron. Modern wild and transplant shells had similar $\delta^{13}C$ ($F_{1,21} = 1.14$, $p = 0.31$).

Percent N was lower in ancient than modern wild shells ($F_{1,45} = 94.7$, $p < 0.001$; Fig. 3b, Table 4), with no significant difference among sites ($F_{2,45} = 0.28$, $p = 0.76$). Modern wild and transplant shells had similar %N content ($F_{1,22} = 0.09$, $p = 0.77$, Fig. 3d), but %N differed by site for these shell types (Heron, North Rigolets > Cumbest; $F_{2,22} = 12.2$, $p < 0.01$, Tukey's < 0.05). Among ancient shells, shell age since deposition (550–2420 years old, Table 2) did not have a significant effect on shell %N (linear regression, $F_{1,23} = 1.85$, $r^2 = 0.07$, $p = 0.19$). Ancient shells also had significantly lower organic %C ($\%C_{org}$) than shells from modern wild oysters ($F_{1,45} = 115$, $p < 0.001$), and there were significant site differences for $\%C_{org}$ ($F_{2,45} = 5.13$, $p = 0.01$, Heron > Cumbest). Ancient shells had significantly higher organic C:N than modern shells ($F_{1,41} = 110$, $p < 0.001$), and site-related differences in C:N ($F_{1,41} = 5.38$, $p = 0.01$) were driven by Heron shells with higher C:N than Cumbest (Table 4). Modern wild and transplant shells did not have different C:N ($F_{1,20} = 3.67$, $p = 0.08$), but shells from different sites had consistently different C:N ($F_{2,20} = 14.4$, $p < 0.001$, Heron > North Rigolets > Cumbest). There was no relationship between C:N and $\delta^{15}N$ values in ancient or modern shell.

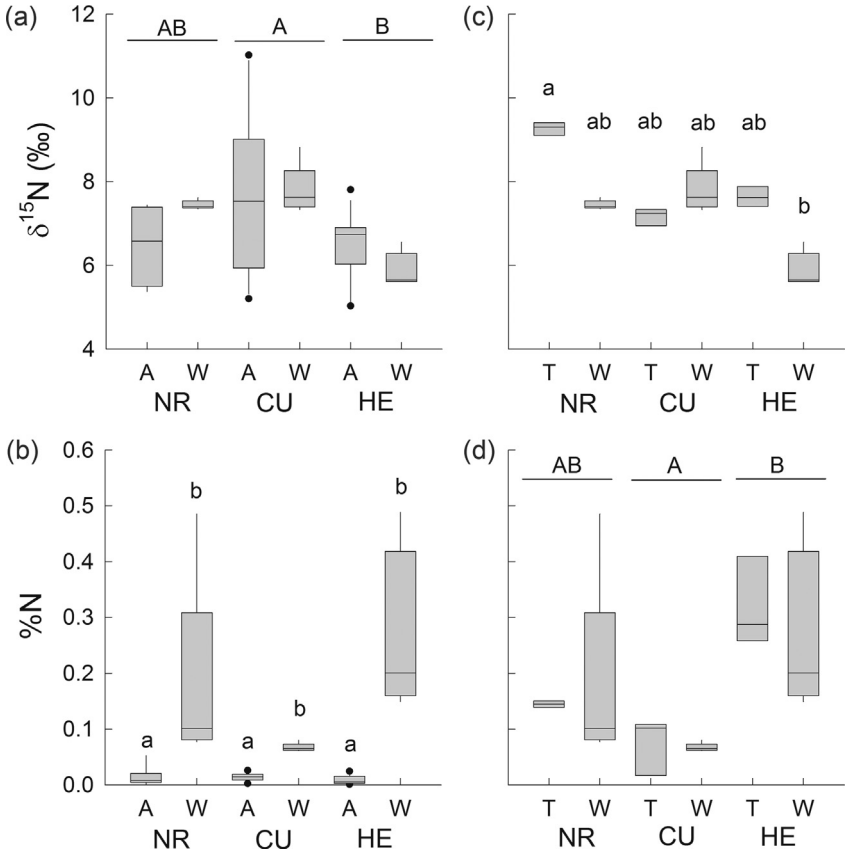

Fig. 3. Shell $\delta^{15}N$ values (top panels) and % N (bottom panels) for each shell type (ancient = A, wild = W, transplant = T) at Bayou Cumbest (CU), Bayou Heron (HE), and North Rigolets (NR). Similar values are shown by a horizontal bar and capital letters (A or B representing end member N sources; AB representing mixed N sources); similar values due to site interaction are shown by lower-case letters (a or b; Tukey's HSD, $\alpha = 0.05$).

3.2. Environmental influences on modern shell $\delta^{15}N$ values

In modern oysters, there were consistent relationships among isotope values in SPM, oyster tissue, and shells that corresponded to known land use in the watershed for each site, such as urbanization and wastewater inputs (Table 5). Mean $\delta^{15}N$ values in suspended particulate matter (SPM) from each site during each study period (2011 or 2012-13) predicted both wild and transplant $\delta^{15}N$ values in oyster adductor muscle (wild: $r^2 = 0.50$, $p < 0.01$; transplant: $r^2 = 0.62$, $p < 0.01$, Fig. 4). SPM $\delta^{15}N$ also had a significant positive relationship with $\delta^{15}N$ values in shell of modern transplant oysters, but not in shells of modern wild oysters (transplant: $r^2 = 0.67$, $p < 0.03$). Among transplanted oysters, $\delta^{15}N$ values in shell were not significantly different from $\delta^{15}N$ values in soft tissues (Tukey's, $p = 0.72$). Soft tissues were significantly enriched 3.7 ± 0.3‰ and shells were enriched 3.5 ± 0.3‰ (mean ± SE) in transplanted oysters compared to mean SPM $\delta^{15}N$ values (Tukey's, $p < 0.0001$ for both comparisons, Fig. 4).

$\delta^{15}N$ values in modern transplant shells, tissues, and SPM varied by site relative to land use and water column nutrient concentrations. Overall, sites that had significantly higher $\delta^{15}N_{shell}$ (one-way ANOVA, $F_{7,40} = 15.6$, $p < 0.001$; Tukey's HSD) were more urbanized, with higher watershed impervious area, population densities, and wastewater inputs (Fig. 5, Table 5, Darrow, 2015), but less marsh area (Fig. 1), and a history of land-use change through time (Bayou Chico, Bayou la Batre, Bangs Lake) compared to less urbanized sites (North Rigolets, Bayou Cumbest, Bayou Heron; Darrow et al., 2016). Shell, soft tissue, and SPM $\delta^{15}N$ values had a positive logarithmic relationship with mean water column nitrate concentration across sites (Fig. 5a), and $\delta^{15}N$ in SPM and tissue had a negative linear relationship with mean dissolved organic nitrogen (DON) concentration across sites (Fig. 5b).

3.3. Effects of stable isotope analysis methods

3.3.1. Method effects

There was no significant difference in $\delta^{15}N$ values among methods within a shell type (midden or modern wild), but there was a higher amount of variability in results from the filter method (Fig. 6). Due to instrument limitations of the bulk method with respect to sample size, smaller sample masses of shell powder were processed than acidification methods, hence, a greater proportion of the bulk samples were rejected due to the analyzed N mass being <20 μg. However, when %N data from shell organic matter was scaled up to the mass of shell powder originally acidified,

Table 4
Oyster shell N and C isotope values, percent organic N and C by mass and molar organic C:N in ancient (midden) and modern (Wild and Transplant) oysters from Grand Bay, MS. Data from all three methods (bulk, centrifuge, filter) were combined when results among sampling methods did not statistically differ. All values are mean ± SE. The %N in whole shells was calculated by scaling up %N in EA-IRMS samples (organic matter only) to the mass of shell powder (organic + inorganic) originally analyzed. Comparisons of shell %N to samples measured on an elemental analyzer (EA) alone, and to previous studies using primarily the EA.

Shell type	Site	*n*	$\delta^{15}N$ (‰)	%N	$\delta^{13}C$ (‰)	%C	C:N	Shell %N, other methods
Ancient	NR	8	6.47 ± 0.32	0.015 ± 0.006	−22.32 ± 0.24	0.050 ± 0.016	8.31 ± 1.07	Midden: 0.045 ± 0.016 (current study, EA); Aged 7 y: 0.15 ± 0.01 (Kellogg et al., 2013, 2014, EA)
	CU	12	7.73 ± 0.56	0.014 ± 0.002	−22.89 ± 0.26	0.053 ± 0.009	7.54 ± 0.38	
	HE	12	6.47 ± 0.22	0.009 ± 0.002	−23.42 ± 0.27	0.045 ± 0.011	10.36 ± 0.38	
Wild	NR	5	7.45 ± 0.05	0.176 ± 0.078	−21.91 ± 0.40	0.623 ± 0.287	4.42 ± 0.07	0.105 ± 0.005 (current study, EA); 0.21 ± 0.05 (Kellogg et al., 2013, 2014, EA); 0.196 (Hunter and Harrison, 1928, Kjeldahl)
	CU	5	7.79 ± 0.27	0.067 ± 0.003	−23.43 ± 0.30	0.172 ± 0.023	3.89 ± 0.13	
	HE	6	5.89 ± 0.18	0.259 ± 0.077	−23.23 ± 0.57	1.509 ± 0.307	4.77 ± 0.02	
Transplant	NR	3	9.27 ± 0.09	0.145 ± 0.003	−21.08 ± 0.11	0.504 ± 0.009	4.07 ± 0.09	Adult: 0.26 ± 0.01; Juvenile: 0.46 ± 0.01 (Dalrymple and Carmichael, 2015, EA)
	CU	3	7.18 ± 0.11	0.076 ± 0.030	−22.78 ± 0.33	0.255 ± 0.098	4.01 ± 0.13	
	HE	3	7.64 ± 0.14	0.318 ± 0.046	−23.63 ± 0.02	1.210 ± 0.175	4.42 ± 0.03	

Table 5
Watershed attributes (% impervious surface, population density, wastewater influence, water column nutrient concentrations) at each site where modern oysters were transplanted. Wastewater influence is characterized as point (P) and non-point (NP) wastewater sources, and categorical fecal indicator densities (high or low). Nutrient concentrations (mean ± SE) for each site were sampled biweekly to monthly for the same time periods that transplant experiments took place (Jun - Sep 2011, Jun 2012 - May 2013).

Site	Impervious %*	Population density (# ha^{-1})*	Wastewater influence†	NO_3^- (μM)	NO_2^- (μM)	NH_4^+ (μM)	DON (μM)	PO_4^{3-} (μM)
BB	2.07	0.86	P – high	3.81 ± 1.98	0.05 ± 0.02	8.43 ± 2.46	31.4 ± 1.9	3.10 ± 2.07
CH-1	27.6	8.1	NP – high	1.98 ± 0.39	0.25 ± 0.07	8.94 ± 2.15	35.9 ± 1.8	12.41 ± 9.75
CH-2				2.57 ± 1.68	0.34 ± 0.21	6.54 ± 2.46	25.7 ± 6.2	7.25 ± 0.98
BA-1	8.51	0.036	NP – low	1.02 ± 0.37	0.04 ± 0.01	4.23 ± 0.75	40.3 ± 2.4	10.43 ± 8.03
BA-2				0.52 ± 0.11	0.05 ± 0.03	2.03 ± 0.70	47.5 ± 2.9	0.50 ± 0.07
CU-1	1.09	0.088	NP – low	0.44 ± 0.09	0.13 ± 0.05	4.75 ± 0.76	38.9 ± 1.7	0.84 ± 0.29
CU-2				0.72 ± 0.27	0.25 ± 0.12	5.41 ± 1.61	48.7 ± 3.4	0.26 ± 0.03
HE-1	0.63	0.057	NP – low	0.63 ± 0.20	0.04 ± 0.01	3.50 ± 0.57	36.4 ± 2.0	0.34 ± 0.05
HE-2				0.08 ± 0.05	0.76 ± 0.45	5.03 ± 1.48	46.9 ± 4.4	0.27 ± 0.06
PC	1.51	0.01	NP – low	0.69 ± 0.19	0.04 ± 0.02	3.85 ± 0.77	31.0 ± 2.1	3.81 ± 3.02

* Darrow et al. (2016).
† Darrow (2015).

midden shells that underwent acidification (centrifuge or filter methods) had less %N recovered than unacidified (bulk) samples ($F_{2,33} = 14.7$, $p < 0.0001$, Fig. 6b). N yields for acidified samples compared to bulk shell were on average 61–75% for modern wild shells (paired *t*-test, $t = -4.92$, $df = 10$, $p = 0.0003$), and 19–22% for ancient shells ($t = -8.65$, $df = 9$, $p < 0.0001$). Shells sampled using the filter and centrifuge methods had similar $\delta^{13}C$, $\%C_{org}$, and organic C:N, while bulk shell could not be sampled for organic C (Table 4).

3.3.2. Method precision

We accepted some samples that had <20 μg N if analytical replicates were within 0.2‰, but noted the number of samples for each treatment that were above this guideline. After rejecting %N data due to poor reproducibility and sample loss during the acidification process (Table 3), we estimated that for ancient shells (low N), 86% of centrifuge samples were quantifiable for %N and between 71 and 75% of filter samples were quantifiable for %N. For ancient shells, the filter method using 500 mg of sample resulted in the highest proportion of samples with N content above the 20 μg threshold for IRMS analytical precision. Fewer 500 mg filter samples were usable for analyses compared to the other methods, however, because residual carbonate resulted in poor within-shell reproducibility of some $\delta^{15}N$ results (Table 3). When 250 mg of ancient shell powder was processed using the filter method, half of the samples were rejected due to poor reproducibility (Table 3). Direct acidification of excess carbonate on filters was unsuccessful due to the high concentration of carbonate relative to the acid strength.

For modern wild shells, all samples processed by centrifuge or filter acidification methods had sufficient N for IRMS analytical precision, while the bulk method resulted in only 46% of modern samples above the recommended 20 μg N per sample. The bulk method also was more problematic due to incomplete combustion and high residual carbonate reducing the quality of $\delta^{15}N$ data, leading to final rejection of 8% of modern bulk samples. After QA/QC was

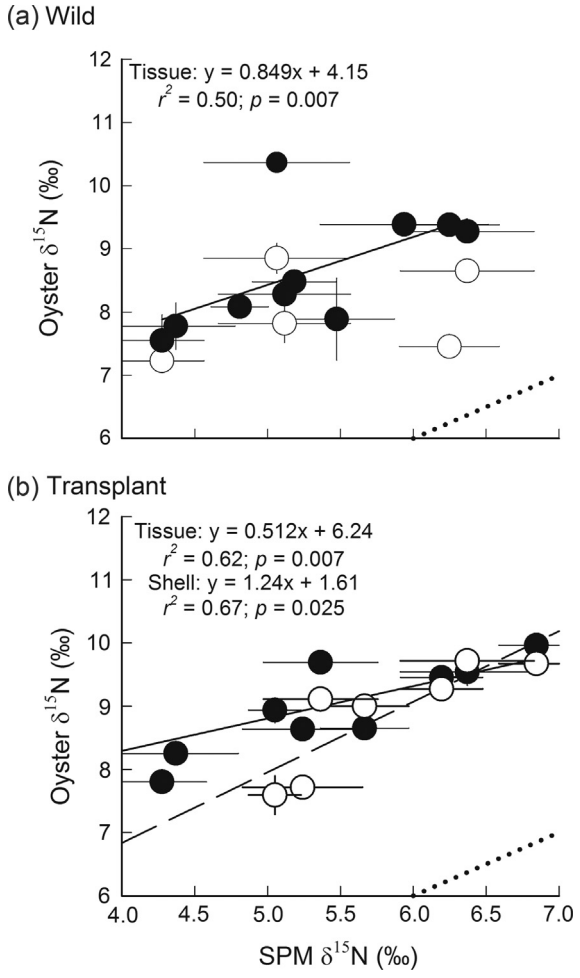

Fig. 4. $\delta^{15}N$ values in tissue (closed circles) and shell (open circles) from modern wild (a) and transplanted (b) oysters compared to values in suspended particulate matter (SPM) (site mean ± SE). Solid and dashed lines show significant linear regression between tissue and shell, respectively, and SPM. Dotted line indicates the 1:1 line of perfect fit.

complete, however, within-shell analytical reproducibility (precision) for $\delta^{15}N$ was best for the bulk method (coefficient of variation, CV = 0.06), followed by acidified-centrifuge method (CV = 0.26), then the acidified-filter method (CV = 0.36). Reproducibility for $\delta^{13}C$ was better for centrifuge (CV = 0.01) than for filter samples (CV = 0.02), and reproducibility for %N was better for bulk (CV = 0.05) and centrifuge samples (CV = 0.06) than filter samples (CV = 0.36).

3.4. Midden N content

Estimated relic N content in oyster shells within Grand Bay middens (remaining after 1000–2000 years) ranged from 410 kg N in midden 22JA564 (HE) to 39,000 kg N in midden 22JA575 (NR) (Fig. 8), with N burial rates ranging from 0 to 5 g N m^{-2} y^{-1} (Table 6). The North Rigolets midden, which was the largest in area, also had the highest shell density of the three sites examined (Table 6), resulting in higher estimated relic N content despite similar %N content in shell compared to other middens (Fig. 3). Estimated N burial rates in middens were comparable to estimated N burial in sediments at all sites except Bayou Cumbest (CU), where sediment N burial exceeded midden shell deposition rates (Fig. 8).

4. DISCUSSION

Through this work, we were able to meet three important objectives: (1) apply stable isotope values in oyster shells as a proxy to better understand ancient (pre-colonial) sources of N to marine systems and changes in N sources through time; (2) improve methods to reliably measure $\delta^{15}N$, organic $\delta^{13}C$, and organic matter content in ancient oyster shells; and (3) apply the most suitable method to determine relic N content as a measure of the potential for middens to provide long-term N sequestration.

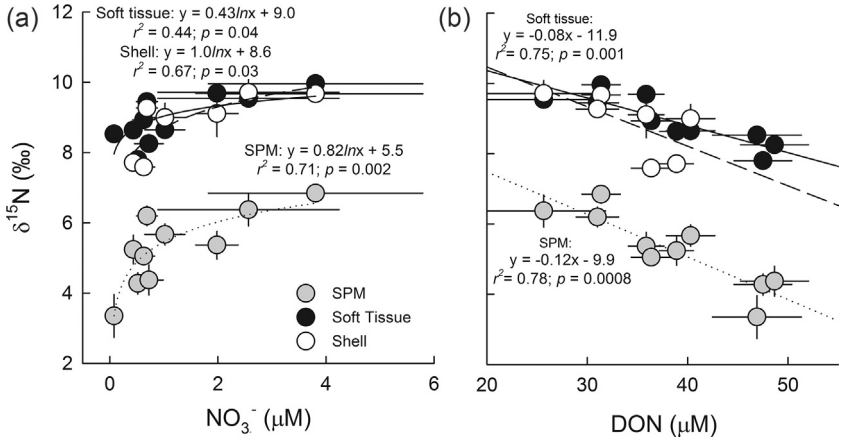

Fig. 5. Mean (± SE) $\delta^{15}N$ in suspended particulate matter (SPM), modern transplant oyster soft tissue, and shell compared to mean (± SE) (a) nitrate and (b) dissolved organic nitrogen (DON) concentrations for each site during transplant experiments.

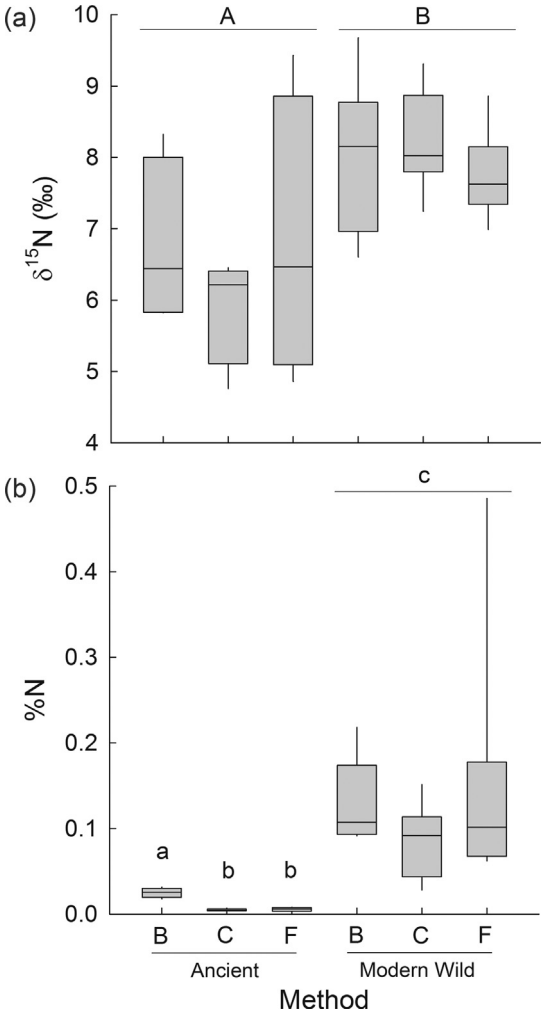

Fig. 6. (a) $\delta^{15}N$ and (b) %N in shell from ancient and wild modern oysters determined using different methods, including the established filter (F), modified centrifuge (C), and bulk shell (B) methods. Similar results are indicated by the same letters (two-way ANOVA, Tukey's HSD, $\alpha = 0.05$).

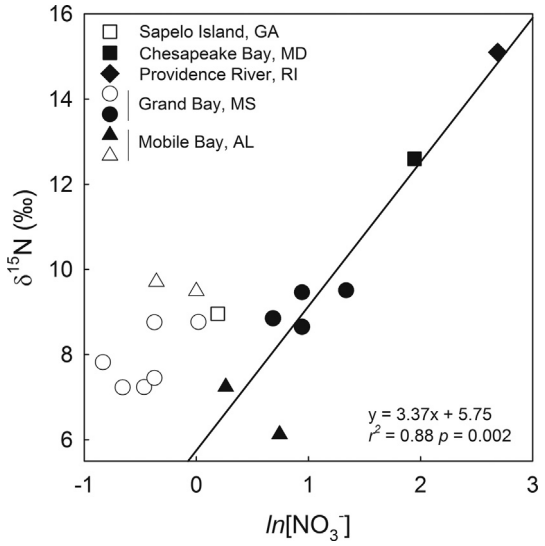

Fig. 7. Oyster shell $\delta^{15}N$ values compared to nitrate concentrations measured in this study (Grand Bay, MS) and others (Kovacs et al., 2010; Black, 2014; Oczkowski et al., 2016). Sites with known wastewater inputs are shown with filled symbols.

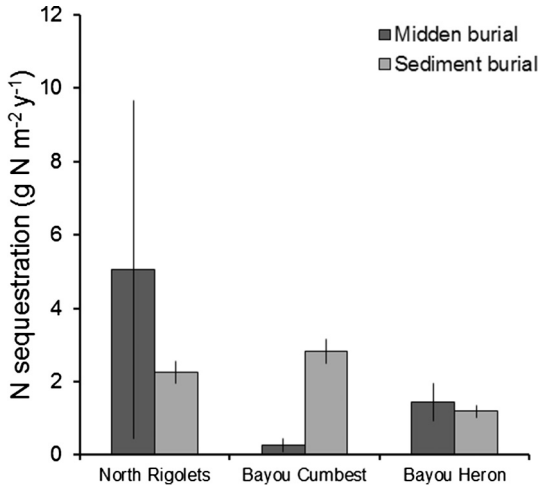

Fig. 8. Estimates of long-term nitrogen sequestration rates at three sites in the Grand Bay estuary, through deposition of oyster shell and retention of relic N in middens (dark bars) and long-term N burial in tidal creek sediments (Darrow et al., 2016).

4.1. Oyster shells as a proxy for ancient N sources

The similarities between $\delta^{15}N$ values in ancient midden and modern wild shells in the Grand Bay system suggest that wild oysters in the area have not had significant changes to $\delta^{15}N_{shell}$ for thousands of years. This finding is consistent with the relatively unurbanized landscape of the Grand Bay National Estuarine Research Reserve (Table 5). Two sites within the Reserve, Bayou Heron and Bayou Cumbest, had oyster shells with $\delta^{15}N$ values characteristic of greater freshwater and marine influences, respectively. Shells from North Rigolets had a range of $\delta^{15}N$ indicating a mixture of N sources, which may result from movement of oysters by native peoples from nearby Heron and Cumbest to the North Rigolets site (cf Fig. 1). Sediment core samples from these sites also indicated $\delta^{15}N$ values and N sources were relatively unchanged for the past 100 years, with no detectable influence of anthropogenic N sources ($\delta^{15}N$ standard deviations ±0.2–0.3 ‰ throughout Heron, Cumbest, and North Rigolets cores; Darrow et al., 2016). In contrast, in the Chesapeake Bay and Providence River (Black, 2014; Oczkowski et al., 2016), modern *Crassostrea virginica* had significantly higher $\delta^{15}N_{shell}$ than ancient oyster shell. Chesapeake Bay and the Providence River have a long history of agriculture, industrialization, and anthropogenic N loading (Zimmerman and Canuel, 2000; Bratton et al., 2003; Oczkowski et al., 2016) compared to Grand Bay, which is in the intensifying or incipient stage of land-use change (Foley et al., 2005). Overall, our results suggest that shells are capable of distin-

Table 6
Estimates of oyster shell-derived nitrogen (N), shell deposition, and N burial rates in three Grand Bay shell middens. Calculations are based on N content of ancient shell at time of excavation (%N measured at each site during this study), which represents relic N remaining in middens. Shell deposition rates are based on ^{14}C-derived dates from >1 excavated level.

Site	Provenience	Midden Vol. (m^3)	Mean shell N density (kg N m^{-3})	Total midden N (kg)	Shell deposition rate (cm y^{-1})	N burial rate (g N m^{-2} y^{-1})
CU (22JA633)	N492 E550	4,500	1.35 (1.02–1.84)	6,600 (4,160–9,050)	0.05	0.38
	N491 E550		1.42 (0.925–2.01)		0.07	0.00
	N491 E563		1.35 (1.06–1.58)		0.04	0.33
HE (22JA564)	N491 E494	1,080	0.853 (0.380–1.49)	920 (410–1,610)	0.46	1.43
NR (22JA575)	N505 E506	18,750	2.79 (1.70–3.88)	25,000 (13,600–39,000)	0.50	5.05
	N495 E478		1.76 (1.09–2.44)			

guishing unaltered from altered N sources across decadal to century timescales.

The reliability of $\delta^{15}N$ as a source tracer depends on knowledge of potential source endpoints and the biochemical reactions that can cause fractionation and variability in $\delta^{15}N$ in a given system. Higher $\delta^{15}N$ in marine organic matter sources depends on NO_3^- concentrations (Altabet and Francois, 1994) and the extent of nitrification–denitrification as N travels from watershed to estuary (Mariotti et al., 1984), and can be overprinted by biochemical alteration after deposition (Thornton and McManus, 1994) or mixing with external N sources such as wastewater. Other studies have found reliable patterns of enriched $\delta^{15}N$ in bone collagen of pre-historic humans (Schoeninger et al., 1983), and in fish, mammals, and birds (Schoeninger and DeNiro, 1984) that consumed a marine-derived diet, compared to a terrestrial diet. Our observations suggest that $\delta^{15}N$ in bivalve shells may also vary with relative to upland or marine influence, along with other well-defined salinity indicators such as $\delta^{13}C$ (McConnaughey and Gillikin, 2008) or $\delta^{18}O$ (Surge et al., 2001). Hence, in pre-colonial bivalves, which were not affected by large-scale wastewater or agricultural N inputs, $\delta^{15}N$ may serve as a proxy for terrestrial or marine-derived N.

Although we did not directly compare ancient and transplanted oysters due to the limited locations of middens, our data corroborate the idea that relationships between ancient and modern oysters are mediated by site-specific factors. Wild and transplanted oysters had similar correlations between shell and soft tissue $\delta^{15}N$ and water column nutrient concentrations, but significant differences in the magnitude of $\delta^{15}N$ values at some sites. SPM $\delta^{15}N$ values differed only slightly between surface and bottom samples at the well-mixed, shallow sites (SPM $\delta^{15}N \pm 0.23‰$, Fig. S2, water depth <1.5 m), but wild oysters growing on the bottom could consume more sediment-derived organic matter than suspended transplanted oysters, resulting in possible differences due to feeding location. Particle selection and/or assimilation rates also may differ between oysters of different ages (Dalrymple and Carmichael, 2015), potentially resulting in higher $\delta^{15}N_{shell}$ values in transplanted (<1.5 years old) than wild oysters (up to 5 years old, Carriker, 1996). Hence, differences in N sources and available foods among sites as well as short-term differences in ontogeny potentially affected $\delta^{15}N_{shell}$ in modern oysters, resulting in greater differences in $\delta^{15}N_{shell}$ among sites for modern oysters than through time (between ancient and modern oysters) in our system.

Accordingly, increased human influence on N sources to the nGOM in recent years was better reflected in modern shells transplanted to sites with a range of land uses. Even at incipient levels of land-use change, sites with higher impervious surface or wastewater inputs outside the Grand Bay Reserve had significantly higher $\delta^{15}N_{shell}$ values than sites within the Reserve (Fig. 7, filled circles). Higher water column mean NO_3- and lower DON concentrations were indicative of higher $\delta^{15}N$ values in modern oyster shell, tissue, and SPM within our study (Table 5, Figs. 5 and 7). Previous studies (*e.g.*, McClelland and Valiela, 1998; Bucci et al., 2007) also found that water column NO_3^- concentrations were correlated with enriched SPM and faunal $\delta^{15}N$ values, and this pattern appears to hold for shell $\delta^{15}N$ values from modern oysters collected from multiple estuaries, including Mobile Bay, AL (Kovacs et al., 2010), Chesapeake Bay, MD, (Black, 2014) and Providence River, RI (Oczkowski et al., 2016). The logarithmic relationship between consumer $\delta^{15}N$ and NO_3^- concentration resembles a Rayleigh-type isotope fractionation model, and may be explained by coupled nitrification–denitrification during the wastewater treatment process resulting in elevated $\delta^{15}N$ of remaining NH_4^+, which likely supports primary production in wastewater-dominated estuaries. In Greenwich Bay, RI, $\delta^{15}NH_4^+$ is high ($14.23 \pm 0.51‰$) and this high $\delta^{15}N$ substrate value explains high $\delta^{15}N$ values of phytoplankton, macrophytes, and shellfish in that estuary (DiMilla et al., 2011).

Although very few studies have examined $\delta^{15}N$ values in DON, or made concurrent measurements of DON concentrations with SPM or consumer $\delta^{15}N$ values, Perakis and Hedin (2002) found that high DON concentrations were typical of pristine wooded watersheds in South America, while high NO_3^- concentrations were more typical of human-influenced wooded watersheds in the northeastern United States. The tidal creeks of Grand Bay have very high DON concentrations compared to other estuaries (reviewed by Sipler and Bronk, 2015), consistent with forest or marsh organic matter sources rather than human sources of N. Correlation between SPM $\delta^{15}N$, oyster tissue and shell $\delta^{15}N$ values (Fig. 3), confirms N in SPM (*i.e.* oyster food) was incorporated into shell organic matrix (Geist et al., 2005; Kovacs et al., 2010). Assuming ancient shell $\delta^{15}N$ values reflect ancient SPM, our data suggest estuarine

waters in the Grand Bay system up to 2000 years ago had similar sources and concentrations of dissolved nitrogen species as the present day.

4.2. Effects of diagenesis

Diagenesis of ancient oyster shell did not affect interpretation of $\delta^{15}N$ values. Although extensive studies have not been done on diagenesis of shell-bound N, we used similar criteria to determine long-term alteration of $\delta^{15}N$ values as studies on ancient bones and teeth (Ambrose, 1990), bird shell (Johnson et al., 1998), and corals (Marion et al., 2005). Ancient shells had significant reductions in %N and $\%C_{org}$, and increases in organic C:N compared to modern shell, indicating diagenesis. C:N alone was not used as a screening tool for diagenesis as others have done for bones and teeth because all ancient samples had higher C:N than modern samples. The lack of a relationship between C:N and $\delta^{15}N$ values in ancient or modern shell indicates that despite N loss, bulk $\delta^{15}N$ values were unaffected by diagenesis. A study replicating effects of diagenesis on $\delta^{15}N$ values in ostrich egg shell (Johnson et al., 1998) found that despite loss of specific amino acids and shifts in compound-specific $\delta^{15}N$, there was little to no change in bulk $\delta^{15}N$ of shell organic matter. Preliminary scanning electron microscopy on archaeological oyster shells of similar ages as the shells in our study found no quantitative differences in intracrystalline organic matter between ancient and modern shells (Black, 2014). Hence, although we detected diagenetic effects on organic matter in ancient shell, these changes did not limit our ability to apply $\delta^{15}N$ values to trace N sources in ancient shells because we found no evidence of diagenesis preferentially degrading or resulting in loss of depleted forms of $\delta^{15}N$. Among ancient shells, we found no significant loss of N with shell age and no difference in stable isotope values within a site.

One potential cause of diagenesis or sample contamination among shells at our sites was fire. Roasting oysters over a wood fire was a common practice, and shells discarded in middens may have been cooked, potentially altering their organic composition (Jackson, 2015). We found examples of $\delta^{15}N$ values being both enriched and depleted in correspondence with archaeological fire features (Table 2). Three shells from one level at site Cumbest (Cat #219, 43–50 cm depth) had high $\delta^{15}N$ (11.6–15‰) and organic C:N values, and were excavated from a level 10 cm below a roasting facility feature. A single shell from site North Rigolets (Cat #18, 100–120 cm depth) had very low $\delta^{15}N$ (−0.25‰), 6‰ depleted compared to other shells from the same site and level (Table 2). While this shell had no visual evidence of burning and adequate N and C for analysis, it had an odor like a wood fire, and the stable isotope values of this shell were similar to signatures in hardwoods such as oak (*Quercus alba* $\delta^{15}N$ ~−1.52‰, McLauchlan and Craine, 2012). This midden site is a shell heap that is proposed to be an oyster processing site rather than a residential site (Jackson, 2015), making it is possible that a fire for roasting oysters was present here. Although one previous study found slight positive changes to oyster shell $\delta^{15}N$ values from experimental heating (Black, 2014), we propose that diagenetic alteration due to repeated heating may elevate $\delta^{15}N_{shell}$ values, but that in some cases, wood ash or smoke may infiltrate the shell matrix and decrease $\delta^{15}N_{shell}$.

4.3. Method effectiveness

4.3.1. Stable isotope values

All methods were acceptable for analyzing modern shell $\delta^{15}N$, although low total N content in bulk shell samples led to more problems with reduced quality of $\delta^{15}N$ data as shown by the variability of results. Acidification may not be necessary, however, if the objective is obtaining only $\delta^{15}N$ and not organic $\delta^{13}C$ values from modern shell, and instrumentation such as a carbon trap is available to facilitate analysis. For ancient midden shells there was no significant difference among methods for $\delta^{15}N$, but the filtration method had the greatest variability. The centrifuge method best avoided carbonate interference because samples were consistently, completely acidified. For ancient shells, a larger proportion of centrifuge samples had enough N for analysis (78%) compared to filter samples (50%) because acidification allowed N to be better concentrated. Hence, our recommendation for accurate measurements of $\delta^{15}N$ values in oyster shell is to use any of the three methods for modern shells (with careful screening of data), but to use the centrifuge method for ancient shells, comparisons among ancient and modern shells, and any cases where determination of $\delta^{13}C$ values is desired. While it is not necessary to run samples on the EA-IRMS in dual mode for both $\delta^{15}N$ and organic $\delta^{13}C$, additional diagnostic information is given about data quality (i.e.; completeness of combustion) and organic matter sources (i.e.; freshwater v. marine) by analyzing $\delta^{13}C$ values and organic C:N.

4.3.2. Organic matter content

For determination of %N, we estimated that 100% of samples using the bulk method would be quantifiable because analysis was directly performed on unprocessed samples, assuming sufficient sample for analysis. Similarly, although the centrifuge method had more steps, all reactions and manipulations occurred within the centrifuge tube, reducing the potential for error due to sample loss or handling compared to the filter method. To increase N recovery from ancient shells, increasing shell powder mass for centrifuge samples may be possible, but was not tested in this study. Increasing shell powder mass slightly for bulk samples also may be possible, but only up to 6 mg C, depending on the instrument, and will likely increase the chance of C interference, making this option not viable for most low-N high-C ancient shells. While methods should continue to be refined, to have the largest number of consistent results from ancient oyster shell, our recommendation is to use the centrifuge method with at least 250 mg shell powder (this amount is expected to vary among bivalve species relative to initial organic matter content and individual rates of diagenetic N loss). As instrument technology continues to improve, we hope that it will be possible to analyze ancient shell for $\delta^{15}N$ without acidification because ancient shell (in which N is already

most scarce) seems to lose more N from this process than modern shell.

4.3.3. Shell cleaning and preparation

Chemical cleaning of samples to remove intercrystalline organic matter is a method that has been predominantly used to examine $\delta^{15}N$ values of diatom (*e.g.,* Sigman et al., 1999), foraminiferal (*e.g.,* Ren et al., 2009), and sometimes, coral skeleton organic matter (Wang et al., 2014). Chemical cleaning has not been used as a standard treatment for bivalve shell prior to $\delta^{15}N$ determination (Carmichael et al., 2008; Oczkowski et al., 2008; Kovacs et al., 2010; Versteegh et al., 2011; Black, 2014). A small-scale trial of effects of a chemical cleaning method similar to that used by Ren et al. (2009) showed that chemical cleaning resulted in low N recovery and high fractionation of $\delta^{15}N$ compared to mechanical cleaning alone (Table S1). Our tests indicate that chemical cleaning methods used for foraminifera and corals are too harsh for ancient bivalve shell. More study should be done to determine whether mechanical or chemical cleaning may be most appropriate for different types of biogenic carbonate.

4.4. N content in ancient shell

The %N we measured in ancient oyster shell was lower than values previously reported for modern oyster shells (Table 4), with N losses of 71–88% in ancient compared to modern wild oyster shells (Fig. 3b). In ancient shells, EA-IRMS methods underestimated shell %N compared to a subset of the same samples run on bulk shell using an EA alone, whereas modern shell %N was within the range of EA samples and other published values for oyster shell (Table 4). Kellogg et al. (2013, 2014) found that oyster shells aged 7 years had only 71% of fresh oyster shell N (0.15 and 0.21%N, respectively), indicating that shell N is lost rapidly. In midden shells, we found no significant effect of shell age (radiocarbon date) on %N, indicating that most shell N loss occurred within the first 500 years after oyster death. In preliminary trials, we found that the marginal edge of modern shells, including the periostracum, had significantly higher %N and different $\delta^{15}N$ values than the interior of the shell, but we did not find this difference in ancient shells (Fig. S3). While we avoided the periostracum, Kellogg et al. (2013, 2014) and Dalrymple and Carmichael (2015) included this part of the shell in their shell %N measurements. We propose that degradation of the periostracum is a major cause of N loss from dead oyster shells.

Oyster shell N may be considered a long-term sink of organic N in estuaries. The mass of N sequestered in middens, estimated at thousands of kilograms of shell-derived N, is substantial, even after thousands of years of decomposition, due primarily to the massive size and densely packed shells of some middens. We estimated shells retained 12–29% of their original N after hundreds to thousands of years, and that this N may be buried at rates comparable to sediment N burial. Given that we sampled only oyster shells, and shell middens are found globally to contain a variety of other mollusc shells and biogenic material (Waselkov, 1987), our estimates of N sequestration in middens is likely conservative, and the potential is high for middens to serve as a long-term global coastal sink for relic N from many species.

5. CONCLUSIONS

$\delta^{15}N$ values in oyster shells suggest that N sources to the Grand Bay estuary system in the nGOM have changed little during the past 2000 years. Despite prevalent diagenesis of organic matter in ancient shells and loss of N through time as shells age, determination of $\delta^{15}N$ values in oyster shells from middens was possible when appropriate methods were applied. Midden oyster shell $\delta^{15}N$ and $\delta^{13}C$ values were site-specific, and results support the conclusions by Jackson (2015) that native people living on the Grand Bay estuary discarded oysters within the same local tributaries where they were collected, and that relative contributions of marine and freshwater-derived N to sites within the reserve boundaries did not substantially shift during this time. In the present day, low $\delta^{15}N$ values in SPM, oyster tissue, and shells were also related to low NO_3^- and higher DON concentrations, which are commonly associated with sites less altered by anthropogenic influence. Our data suggest that these water column attributes also were typical in Grand Bay during pre-colonial times. In recent years, as urbanization has encroached on the Grand Bay system from surrounding areas, modern oyster shells at urbanized sites outside the Reserve system have recorded these changes. Further research at middens near urbanized areas or other sites with known wastewater inputs will be useful to further calibrate differences in oyster shell $\delta^{15}N$ values between ancient and modern shells due to land-use change. This study demonstrates the usefulness of midden oyster shells to assess baseline conditions in estuaries over long time scales by demonstrating that midden shells can be an indicator of pre-historic N source to estuaries. Shell middens also may represent a substantial but previously uncharacterized N sink in estuaries around the world where they are found. To include middens in N budgets, measurements of N content in preserved specimens could be made during archaeological investigations. N budgets for present-day oyster reefs could also include quantification of N sequestration in oyster shells and other biogenic material (Kellogg et al., 2013). When coupled with stable isotope analyses of N sources, these values could substantially refine our understanding of N movement and fate in coastal waters.

AUTHOR CONTRIBUTIONS

RHC, ESD, and CFTA originally formulated the idea; HEJ and ESD conducted fieldwork; ESD developed methodology, analyzed samples, and conducted statistical analyses; ESD and RHC wrote the manuscript; CFTA and HEJ provided editorial advice.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2016.12.023.

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