



Nitrogen isotopic analysis of carbonate-bound organic matter in modern and fossil fish otoliths

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Abstract

The nitrogen isotopic composition ($\delta^{15}\text{N}$) of otolith-bound organic matter (OM) is a potential source of information on dietary history of bony fishes. In contrast to the $\delta^{15}\text{N}$ of white muscle tissue, the most commonly used tissue for ecological studies, the $\delta^{15}\text{N}$ of otolith-bound OM ($\delta^{15}\text{N}_{\text{oto}}$) provides a record of whole life history. More importantly, $\delta^{15}\text{N}_{\text{oto}}$ can be measured in contexts where tissue is not available, for example, in otolith archives and sedimentary deposits. The utility and robustness of otolith $\delta^{15}\text{N}$ analysis was heretofore limited by the low N content of otoliths, which precluded the routine measurement of individual otoliths as well as the thorough cleaning of otolith material prior to analysis. Here, we introduce a new method based on oxidation to nitrate followed by bacterial conversion to N_2O . The method requires 200-fold less N compared to traditional combustion approaches, allowing for thorough pre-cleaning and replicated analysis of individual otoliths of nearly any size. Long term precision of $\delta^{15}\text{N}_{\text{oto}}$ is 0.3‰. Using an internal standard of Atlantic cod (*Gadus morhua*) otoliths, we examine the parameters of the oxidative cleaning step with regard to oxidant (potassium persulfate and sodium hypochlorite), temperature, and time. We also report initial results that verify the usefulness of $\delta^{15}\text{N}_{\text{oto}}$ for ecological studies. For three salmonid species, left and right otoliths from the same fish are indistinguishable. We find that the $\delta^{15}\text{N}_{\text{oto}}$ of pink salmon (*Oncorhynchus gorbuscha*) is related to the size of the fish for this species. We find that intra-cohort $\delta^{15}\text{N}_{\text{oto}}$ standard deviation for wild pink salmon, farmed brown trout (*Salmo trutta*), and farmed rainbow trout (*Oncorhynchus mykiss*) are all 0.4‰ or less, suggesting that $\delta^{15}\text{N}_{\text{oto}}$ will be valuable for population-level studies. Lastly, our protocol yields reproducible data for both $\delta^{15}\text{N}_{\text{oto}}$ and otolith N content in 17th century Atlantic cod otoliths. We find that 17th century cod are approximately 2‰ higher than modern cod, arguably consistent with either the larger size of the otoliths (and thus inferred for the fish) or with changes in baseline (primary producer) $\delta^{15}\text{N}$ in the modern coastal ocean compared to the past. All told, the results of this study bode well for the utility of otolith-bound $\delta^{15}\text{N}$ for investigating the environment and ecology of modern and past fish.

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1. INTRODUCTION

The N isotopic content of fish tissues is a widely used tool for determining the relative trophic position of consumers in marine food webs, distinguishing among consumer populations with different feeding or migratory

habits, and identifying the relative contributions of likely prey species when used in conjunction with stomach content data (Hobson, 1999; Phillips, 2001; Parnell et al., 2010; Boecklen et al., 2011; McMahan et al., 2013). However, the white muscle tissue $\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{\text{wmt}}$) commonly used for ecological studies provides a limited temporal scope of months to years and can only be used for modern fish (e.g., Hesslein et al., 1993; Logan et al., 2006; Madigan et al., 2012). In contrast, fish otoliths (“ear stones”), calcareous biominerals in the fish’s inner ear, continuously precipitate calcium carbonate onto an organic matrix over the course of the fish’s life. As otoliths are metabolically inert and start forming when a fish is one day old (Pannella, 1971; Campana and Neilson, 1985; Pereira et al., 1995), otoliths record the chemical and biological information about fish and their environment over their full lifespans (with a few caveats; see Mosegaard et al., 1988; Barber and Jenkins, 2001). Moreover, the preservation of otoliths in sediments, archaeological deposits, or historical archives offers the possibility of studying the lives and environments of past fish (Ivany et al., 2000; Rowell et al., 2010; Gierl et al., 2013).

Otoliths are used for determining age in fishes; over one million fish are aged each year by fisheries biologists for the purposes of fisheries stock assessments and biological studies (Campana and Thorrold, 2001). The field of otolith microchemistry uses the concentrations of trace elements (e.g., Mg, Mn, Fe, Sr, Ba) or isotopes (e.g., $\delta^{18}\text{O}$, $\delta^{13}\text{C}$, $^{87}/^{86}\text{Sr}$, $\Delta^{14}\text{C}$) in the mineral fraction of otoliths over years of the fish’s life or among different fish groups or individuals to reconstruct fish movement or origin, largely based on the chemical “fingerprints” of different estuaries, rivers, or oceanic provinces (Kalish, 1989; Campana and Thorrold, 2001; Sturrock et al., 2012). Importantly, most of these studies focus on the chemistry of the mineral matter (CaCO_3) as opposed to that of the organic matter (OM) (with the important exceptions of Vandermyde and Whitlege, 2008; Rowell et al., 2010; McMahan et al., 2011a,b, 2012; Grønkvær et al., 2013; Sirot et al., 2017). The annual growth bands (“annuli”) that allow for enumeration of fish age result from varying ratios of organic-to-mineral content, with periods of faster somatic growth corresponding to otolith regions with a lower concentration of OM. The OM is the substrate for $\delta^{15}\text{N}$ measurements in otoliths.

Efforts to measure otolith $\delta^{15}\text{N}$ have been limited by the small concentrations of OM in otoliths (usually <1–10% by mass; Degens et al., 1969; Borelli et al., 2001). Previous studies ranged from distinguishing between agricultural and pristine watershed origin (Vandermyde and Whitlege, 2008), to investigating prehistoric trophic relationships (Rowell et al., 2010), to identifying the diet of Atlantic cod (Grønkvær et al., 2013). Most studies required large or multiple otoliths to obtain a single measurement, due to their use of the well-tested but relatively low-sensitivity approach of combustion of OM to N_2 (Vandermyde and Whitlege, 2008; Rowell et al., 2010; Grønkvær et al., 2013; Sirot et al., 2017). Grønkvær et al. (2013) and Sirot et al. (2017) include a dissolution centrifugation step for separation of the water-soluble and

-insoluble fractions of OM prior to combustion to N_2 and subsequent isotope analysis.

In addition to the challenge of the low concentration of OM in otoliths, the fish-native origin and isotopic preservation of the OM must also be assured. In fossil otoliths, diagenetic processes have the potential to cause loss of N-containing components of OM. Loss of N-containing components is frequently associated with isotopic fractionation that results in higher $\delta^{15}\text{N}$ of the remaining OM (Macko et al., 1986; Gannes et al., 1998; Hannides et al., 2013), although $\delta^{15}\text{N}$ can also be lowered and/or simply made more variable under some conditions (Altabet, 1988; Altabet et al., 1991; Lehmann et al., 2002; Tremblay and Benner, 2006; Robinson et al., 2012). Additionally, exogenous N may be added to sedimentary materials during their accumulation, and some of this exogenous N may be mobile in the diagenetic setting. Thus, diagenesis can introduce uncertainty in the interpretation of N isotope measurements in terms of past environmental, ecological, or physiological processes. Studies of fossil carbonate-bound OM in other biogenic structures such as coral, foraminifera, and ostracods have found a preliminary cleaning step to be essential for the removal of altered endogenous OM and exogenous OM (e.g., Ingalls et al., 2003; Ren et al., 2009; Bright and Kaufman, 2011; Wang et al., 2014). Previous work on the $\delta^{15}\text{N}$ of fossil otoliths has generally not taken steps to address or avoid diagenetically altered or exogenous OM (Rowell et al., 2010), in part because cleaning techniques further reduce the amount of otolith-native OM available for isotopic analysis. One goal of the current study was to investigate the necessity and efficacy of a pre-cleaning treatment to remove diagenetically altered and exogenous N so as to leave only otolith-native N for $\delta^{15}\text{N}$ analysis.

Lastly, we demonstrate the application of otolith N isotopic composition for ecological and oceanographic studies. The $\delta^{15}\text{N}$ of metazoans records two factors: the $\delta^{15}\text{N}$ of the primary producers at the base of the food web and the trophic level of the organism.

The $\delta^{15}\text{N}$ of the primary producers at the base of the food web, often referred to as baseline $\delta^{15}\text{N}$, is controlled by large scale factors such as the $\delta^{15}\text{N}$ of nitrate supply to the euphotic zone (Ren et al., 2009, 2012) and the degree of nitrate consumption by phytoplankton (Wada and Hattori, 1976; Altabet et al., 1991; Altabet and Francois, 1994), as well as by more specific factors such as the phytoplankton forms that ultimately support the heterotrophic species being studied (Fawcett et al., 2011, 2014). Differences in primary producer $\delta^{15}\text{N}$ are imprinted on primary consumers, for example copepods, and subsequently propagate up the food chain (Hobson, 1999; McMahan et al., 2013; Dunton et al., 2017). Differences in baseline $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ are used to track marine migrations of species if animals reside in isotopically distinct environments for long enough for their tissues to record the isotopic signal (Schell et al., 1998; Schell, 2001; Newsome et al., 2010; McMahan et al., 2013).

The trophic factor can be summarized by the aphorism, “you are what you eat, plus a few per mille” (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Macko et al.,

1986). This pattern is due to preferential excretion of ^{14}N relative to ^{15}N , leaving tissues enriched in the heavier isotope by an average of 2–5‰ relative to diet (Minagawa and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post, 2002; Vanderklift and Ponsard, 2003), although degree of enrichment can vary among different tissues from the same fish or among species due to differences in protein composition and metabolic routing among tissues (e.g., McMahon et al., 2010; Mohan et al., 2016). Largely due to the trophic effect, the $\delta^{15}\text{N}$ in tissues of metazoans have long been used to reconstruct diet, determine trophic level, and track energy flow through ecosystems (e.g., as reviewed by Boecklen et al., 2011).

To determine whether a given change in fish $\delta^{15}\text{N}$ is the result of diet, baseline, or a combination of the two factors, baseline can be constrained by measuring primary producer or primary consumer $\delta^{15}\text{N}$ from the same geographic region (Post, 2002; Mancinelli et al., 2013). More recently, compound-specific isotope analysis, or CSIA, of individual amino acids (AAs) has been developed to distinguish baseline from trophic level effects on $\delta^{15}\text{N}$ (McClelland and Montoya, 2002; Chikaraishi et al., 2009).

To measure $\delta^{15}\text{N}_{\text{oto}}$, we adapt a method for measuring the N isotopic composition of carbonate-protected organic matter, such as is found in fossilized foraminifera (Ren et al., 2009; Straub et al., 2013) and coral (Wang et al., 2014). This assay requires less N compared to traditional combustion to N_2 and allows for a physical and chemical cleaning that is essential to avoid diagenetic or preparation-related artifacts. Analytical procedures specific to fish otoliths are addressed first. We then turn to questions that determine the potential of $\delta^{15}\text{N}_{\text{oto}}$ as a proxy for fish diet: (1) Is the proxy adequately precise to record differences between individual fish and among fish groups? (2) Is $\delta^{15}\text{N}_{\text{oto}}$ consistent with existing trophic level information? (3) Is $\delta^{15}\text{N}_{\text{oto}}$ robust against diagenetic alteration for historical otolith samples? $\delta^{15}\text{N}_{\text{oto}}$ of historical fishes may provide insight into past food web structure and environmental conditions. This potential application is preliminarily investigated in Atlantic cod (*Gadus morhua*) by comparison of modern and fossil otoliths.

2. METHODS

The $\delta^{15}\text{N}_{\text{oto}}$ method reported here consists of eight steps (Fig. A1): initial cleaning of the whole otolith, crushing of the whole otolith, oxidative removal of “exposed” OM, acid dissolution of the cleaned otolith aragonite, oxidation of the released (“carbonate-bound”) OM to nitrate, quantitative conversion of the nitrate to N_2O , automated extraction, cryogenic and gas chromatography-based purification, and isotopic analysis of the N_2O . The latter steps, which yield isotopic measurements of nitrate, are often summarized as the “bacterial conversion” or “denitrifier method” (Sigman et al., 2001; Casciotti et al., 2002; Mcilvin and Casciotti, 2011; Weigand et al., 2016), which is 200-fold more sensitive than standard approaches involving combustion to N_2 and initiated the N_2O -based approach for isotopic analysis of diverse N forms.

2.1. Preparation and initial cleaning of whole otoliths

Fish (mostly Atlantic cod and salmonids) were obtained from various sources including local fish markets and fish hatcheries (Table 1). Only sagittal otoliths were used. Otoliths used for all experiments below were soaked for 24 h in 10 mL sodium hypochlorite (NaOCl , 10–15% available chlorine) and rinsed three times in deionized water (DIW). They were then transferred to pre-combusted 12 mL borosilicate glass vials and dried for up to 24 h at 30 °C or until completely dry, visually inspected using a microscope to ensure complete removal of tissues, and weighed to ± 0.01 mg.

2.2. Procedures for oxidative cleaning of otolith grains

Cleaned otoliths were crushed and homogenized using a mortar and pestle. The mortar and pestle were cleaned with dust-free spray air and dilute hydrochloric acid (10%) between samples. Grains were ground to 38–150 μm (determined by sieving) unless otherwise stated. Resulting otolith powder was soaked for 24 h in sodium hypochlorite in 15 mL polypropylene centrifuge tubes. Tubes for sodium hypochlorite cleaning were oriented horizontally on a shaker (IBI Scientific) for 24 h. Samples were then rinsed three times with DIW, using centrifugation (3 min at 2900 rcf) to prevent loss of crushed material while removing supernatant between rinses. From this stage forward, the N blank associated with the DIW used for cleaning or included in the persulfate oxidation solution was minimized by a final low-temperature distillation (Savillex Corporation, Minnetonka, MN). This distilled water was used for reagent solutions that are subsequently added to samples. Minimizing the relative contribution of nitrogen from non-sample sources allows for analysis of relatively low N concentrations in otoliths.

After cleaning and rinsing, otolith powder was transferred to pre-combusted 12 mL glass vials, and excess water was removed using pre-combusted glass Pasteur pipettes fitted to a diaphragm vacuum pump. Samples were dried for 12–48 h at 30 °C in a drying oven reserved for low-N samples. Once completely dry, otolith powder was weighed into 4 mL pre-combusted borosilicate glass vials with freshly cleaned and dried Teflon-lined caps. Final masses were 3–4 mg (± 0.01 mg; MettlerToledo) unless otherwise specified.

2.3. Isotopic analysis

Cleaned and dried otolith powder was dissolved, oxidized, and analyzed as in Wang et al. (2014), as summarized here. 50 μL of 4 N Optima grade hydrochloric acid was used to dissolve the CaCO_3 ; sample vials were shaken and visually inspected to ensure complete dissolution of CaCO_3 . One mL of freshly combined persulfate oxidizing reagent (POR) (1 g of 4X recrystallized potassium persulfate and 1 g ACS grade sodium hydroxide into 100 mL DIW) was added to the sample vials and autoclaved for 90 min at 120 °C to convert organic nitrogen to nitrate (Solorzano and Sharp, 1980; Bronk et al., 2000). The reagent mixture

Table 1
Sample locations and dates of otoliths used for $\delta^{15}\text{N}_{\text{oto}}$.

Common name	Latin name	<i>n</i>	Origin	Location (of farm, of excavation, of fish landing)	Type	Sampling date
Brown trout	<i>Salmo trutta</i>	13	Musky Trout Hatchery	Asbury, NJ	Aquaculture (fish farm)	24 July 2014
Rainbow trout	<i>Oncorhynchus mykiss</i>	10	Musky Trout Hatchery	Asbury, NJ	Aquaculture (fish farm)	24 July 2014
Pink salmon	<i>Oncorhynchus gorbuscha</i>	10	Alaska Department of Fish & Game, Cordova, AK	Prince William Sound, Alaska	Wild-caught (commercial)	22 August 2008
Atlantic cod (historical)	<i>Gadus morhua</i>	4	University of Southern Maine, Cornell University	Smuttynose Island, NH	Wild-caught (commercial)	8 June 2010 (excavation)
Atlantic cod (modern)	<i>Gadus morhua</i>	7	Nassau Seafood, Princeton, NJ	Chatham, MA	Wild-caught (commercial)	13 November 2014
Atlantic cod (modern)	<i>Gadus morhua</i>	4	Nassau Seafood, Princeton, NJ	Chatham, MA	Cod otolith standard (CDS)	13 November 2014
Atlantic cod (modern)	<i>Gadus morhua</i>	18	Fishery Biology Program, NOAA Fisheries Northeast Fisheries Science Center (NEFSC)	Georges Bank	Wild-caught (fisheries-independent research survey)	September–November 1981, 1984, 1987, 2013
Atlantic cod (farmed)	<i>Gadus morhua</i>	3	University of Maine Center for Cooperative Aquaculture Research (CCAR)	Franklin, ME	Aquaculture (fish farm)	17 March 2015
Pink salmon	<i>Oncorhynchus gorbuscha</i>	25	ADF&G, Cordova, AK	Prince William Sound, Alaska	Pink salmon standard (PSS)	22 August 2008
Queen snapper	<i>Etelis ocellatus</i>	20	Nassau Seafood, Princeton, NJ	Panama City, Panama	Queen snapper standard (QSN)	2 Nov 2014

is unstable and thus was used immediately after combination. After cooling, precipitate was removed by centrifugation for 4 min at 4600 *rcf*. The supernatant was transferred to new precombusted 4 mL borosilicate glass vials, and the pH was adjusted to 5–7 using aliquots of 6 N HCl.

The concentration of nitrate resulting from the persulfate oxidation step was analyzed by conversion to nitric oxide followed by chemiluminescent detection (Braman and Hendrix, 1989). Per analysis, 10 or 20 nmol N of this nitrate was then quantitatively converted to nitrous oxide (N_2O) using bacterial conversion (Sigman et al., 2001; Weigand et al., 2016). The ratio of ^{15}N to ^{14}N of the N_2O analyte was measured via gas chromatography-isotope ratio mass spectrometry (GC-IRMS) on a purpose-built system for N_2O extraction and purification online to a Thermo MAT253 isotope ratio mass spectrometer (Weigand et al., 2016).

Freshwater solutions of nitrate reference materials were analyzed alongside samples using the bacterial conversion method and were used to calibrate the isotopic composition of samples vs. N_2 in air and to calculate nitrogen concentration. Nitrogen content was calculated using peak area results from the GC-IRMS because it proved more precise than the chemiluminescence measurements described above. For N content, the average standard deviation is 3% of the target N concentration for replication of nitrate reference materials. For $\delta^{15}\text{N}$, the precision of the bacterial conversion and isotopic analysis is $<0.05\text{‰}$ for nitrate ref-

erence solutions (the precision associated with $\delta^{15}\text{N}_{\text{oto}}$ from replicated analyses is discussed extensively below).

2.4. Blank corrections

The nitrogen blank of POR was usually 0.3–1 nmol N. As total oxidized otolith OM was generally 100 nmol N or greater, this amounts to 1% or less of the total N in oxidized otolith samples. The final N content and $\delta^{15}\text{N}$ of oxidized samples were corrected for this POR-associated N blank using organic standards with defined isotopic compositions (glutamic acid reference materials USGS-40 and USGS-41) that were oxidized in parallel with each sample batch to calculate the $\delta^{15}\text{N}_{\text{blank}}$ for each POR batch. Blank corrections were conducted as per (Gelwicks and Hayes, 1990):

$$\delta^{15}\text{N}_{\text{sample}} = \frac{M_{\text{mix}}\delta^{15}\text{N}_{\text{mix}} - M_{\text{blank}}\delta^{15}\text{N}_{\text{blank}}}{M_{\text{mix}} - M_{\text{blank}}} \quad (1)$$

where *M* refers to mass, and the term $M_{\text{sample}} = M_{\text{mix}} - M_{\text{blank}}$, and where M_{mix} , $\delta^{15}\text{N}_{\text{mix}}$, and M_{blank} were measured directly via GC-IRMS. $\delta^{15}\text{N}_{\text{blank}}$ was calculated using a linear regression of USGS40 and USGS41 organic standards. M_{blank} was also measured on the chemiluminescent analyzer. Usually, these blank corrections were calculated to cause less than a 0.1‰ change in the $\delta^{15}\text{N}$ of the sample. Internal otolith standards made of ground pink salmon otoliths (PSS) and cod otoliths (CDS) were run in duplicate

or triplicate with each sample batch to track consistency over time and quantify the long-term precision of the method.

3. METHOD TESTING

Experiments to optimize the cleaning time, investigate the effect of grain size, and test the efficacy of two different cleaning agents were conducted (Table 2). The cod otolith standard (CDS) was made of homogenized sagittal otoliths of four individual Atlantic cod caught on 13 November 2014 and landed in Chatham, MA by cleaning bulk otoliths as above. All four otoliths were combined and ground using an agate mortar and pestle and sieved through sequential sieves in order to size fractionate the cod otolith standard into grain sizes >425 μm , 150–425 μm , and 38–150 μm . This cod standard was called CDS. For the cleaning reagent experiments described below, otolith standards of two other species, queen snapper (*Etelis oculatus*) and pink salmon (*Oncorhynchus gorbuscha*), were also investigated. These two other species were used because they are taxonomically distant from Atlantic cod, helping to ensure that the results of the cleaning tests apply to multiple fish species. Queen snapper standard (QSN) was made with otoliths from fish obtained from Nassau Seafood, Princeton, NJ, combined, ground, and sieved as above. Pink salmon standard (PSS) was made with otoliths provided by the Alaska Department of Fish and Game (ADF&G) from the commercial fishery, combined, ground, and sieved as above.

3.1. Length of time required for sodium hypochlorite cleaning

The effects of cleaning time and temperature were examined using the 150–425 μm grain size of CDS. Otolith powder (3.15–3.75 \pm 0.01 mg) was added to 15 mL polypropylene centrifuge tubes and cleaned using sodium hypochlorite. Samples were either maintained at room temperature (22 $^{\circ}\text{C}$) or heated to 60 $^{\circ}\text{C}$ using a water bath. Centrifuge tubes for both room temperature and heated experiments were shaken vigorously every 6 h. Cleaning times to remove exposed organic matter from the otolith grains were 0.5 h, 1 h, 3 h, 6 h, 9 h, 12 h, 18 h, 24 h, and 36 h at room temperature, and 0.5 h, 1 h, 6 h, 12 h, and 24 h at 60 $^{\circ}\text{C}$. At each time point, sodium

hypochlorite was immediately removed by centrifuging the samples (3 min at 2900 rcf) and gently pouring off the supernatant, followed by rinsing 3X in DIW. After cleaning and rinsing, all otolith powder was prepared and analyzed as above.

3.2. Effect of otolith grain size

The effect of grain size on measured $\delta^{15}\text{N}_{\text{oto}}$ and N content provides insight into the spatial scale at which carbonate-bound N is protected by the mineral fraction. Accordingly, all three grain sizes (>425 μm , 150–425 μm , and 38–150 μm) of the cod otolith standard were cleaned in triplicate by each of two cleaning agents, sodium hypochlorite and persulfate oxidizing reagent (POR) (see Section 3.3 with respect to POR).

3.3. Effect of cleaning agent

To test the efficacy of different cleaning reagents for oxidative removal of exposed OM from otolith grains, either sodium hypochlorite or freshly combined POR was added in 10 mL aliquots to the vials containing otolith powder. POR was described above (Section 2.3) for oxidation of OM for subsequent isotope analysis of the resulting nitrate; here, POR was used to oxidatively clean aragonite grains prior to dissolution. Tests of cleaning agent were conducted for CDS, PSS, and QSN. Only 150–425 μm size fractions were used for each otolith standard. Cleaning with sodium hypochlorite was conducted in 15 mL polypropylene centrifuge tubes as described above (Section 2.2). Cleaning with POR was conducted in pre-combusted 12 mL borosilicate glass vials with individually cleaned Teflon-lined caps, then autoclaved for 90 min at 120 $^{\circ}\text{C}$. POR-cleaned samples were then transferred to 15 mL polypropylene centrifuge tubes. Samples from both sodium hypochlorite and persulfate treatments were rinsed three times in DIW, with a centrifuge step between rinses to concentrate otolith powder and prevent loss of sample, and transferred to precombusted 12 mL glass vials. Extra water was removed using pre-combusted glass pipettes. Samples were then dried in a drying oven at 30 $^{\circ}\text{C}$ for 12 h or until completely dry (usually 12–48 h). Analysis of $\delta^{15}\text{N}_{\text{oto}}$ was conducted as described above (Section 2.3).

Table 2

Tests investigating the analytical precision, ecological accuracy, and preservation of $\delta^{15}\text{N}_{\text{oto}}$.

Analytical ^a	Ecological ^b	Preservation ^c
Long term inter-batch precision	Intra-fish (L vs. R) comparison	Historical vs. modern N content
Cleaning temperature	Fish size (farmed, wild, and historical)	Clean vs. unclean
Cleaning reagent	Intra-cohort $\delta^{15}\text{N}_{\text{oto}}$ variability	Exposure time to oxidizing reagent
Exposure time to cleaning reagent	Different species, same diet	Pulverized vs. intact otoliths
Pulverized vs. intact otoliths	Same species, same diet	
Grain size of pulverized otoliths	Historical vs. modern $\delta^{15}\text{N}_{\text{oto}}$	
Intra-fish (L vs. R) comparison	$\delta^{15}\text{N}_{\text{oto}}$ vs. $\delta^{15}\text{N}_{\text{wmt}}$	

^a Experiments investigating the precision of $\delta^{15}\text{N}_{\text{oto}}$. Refers mostly to experiments conducted with otolith standards.

^b Experiments investigating the origins of $\delta^{15}\text{N}_{\text{oto}}$ e.g., whether signals in otoliths derive from physiological variability, diet, or environment and also whether $\delta^{15}\text{N}_{\text{oto}}$ corresponds to previously confirmed trophic information (e.g., tissue $\delta^{15}\text{N}$, diet, size)

^c Experiments investigating the degree of resistance of $\delta^{15}\text{N}_{\text{oto}}$ to diagenesis

3.4. Effect of cleaning on fossil otoliths

The necessity of removing exposed OM for midden-deposited otoliths was tested. Two broken otoliths (fractured *in situ*, prior to archaeological excavation; Fig. 1) were studied to address whether there was a measurable difference with and without cleaning of their ground powder (Fig. 2). The fossil otoliths were excavated from Smut-

tynose Island, the site of a commercial fishing station that shipped dried cod to European markets during the 17th century (Appendix A for full site description). Fish heads were routinely discarded into trash sites (middens) along with other contemporaneous artifacts including other fish biological remains, ceramics, and pipe stems (Robinson, 2012; Moyer et al., 2015). Because the historical otoliths had been buried in sediments for over 300 years and were

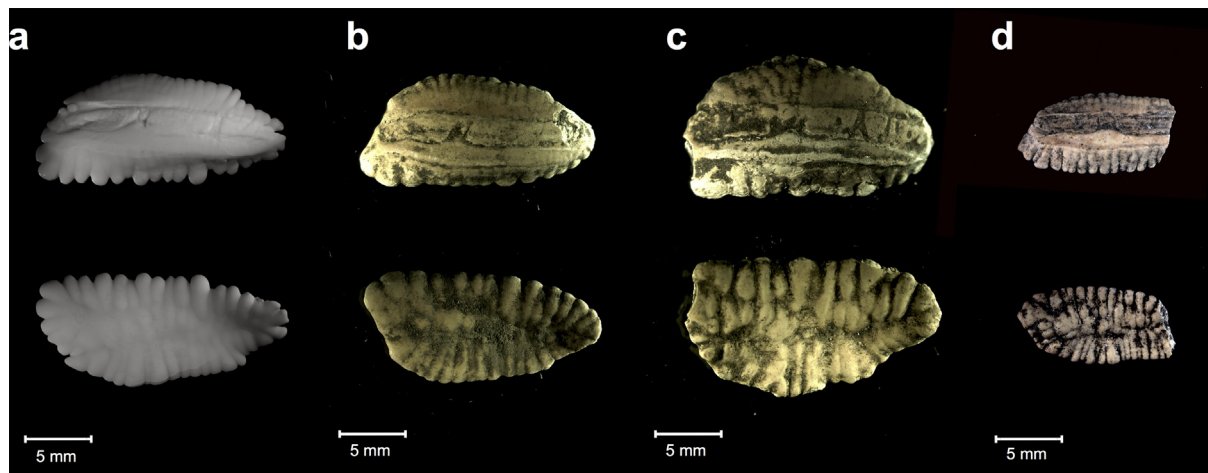


Fig. 1. Modern and 17th century Atlantic cod otoliths from the Gulf of Maine. Modern (a) and 17th century midden-deposited (b–d) Atlantic cod otoliths from the Gulf of Maine. Panel (a) shows a modern otolith (fish age not determined), (b) shows a well-preserved midden otolith of similar size to (a), (c) shows a large (fish age ≥ 9 years) midden otolith that had been chipped *in situ* in the midden, and (d) shows a small (fish age = 3 years) midden otolith that had also been chipped *in situ* in the midden. Otoliths in (c) and (d) are similar in size and preservation (degree of damage) to fossil otoliths A and B, respectively, that were used in the fossil otolith cleaning test (Section 3.4).

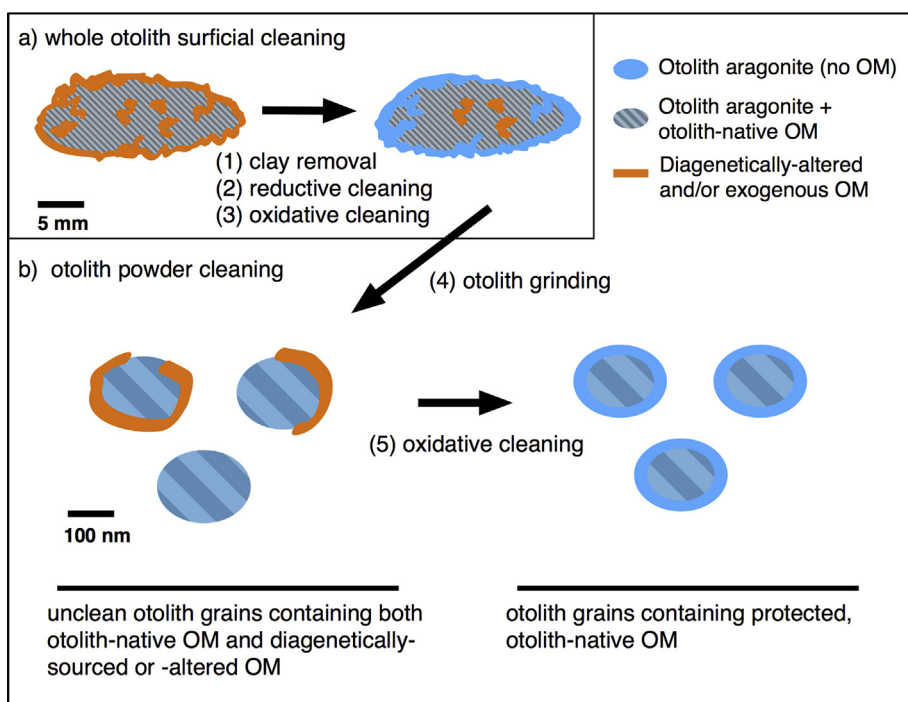


Fig. 2. Diagram of cleaning methods for fossil otoliths. The $\delta^{15}\text{N}_{\text{oto}}$ protocol includes (a) surficial, external cleaning of whole otoliths, followed by (b) crushing and cleaning of otolith grains to remove diagenetically-altered or exogenous N in order to analyze $\delta^{15}\text{N}_{\text{oto}}$ of only otolith-native OM. Orange markings represent diagenetically-altered or exogenous N, blue regions represent aragonite, and blue-black hash marked regions represent aragonite containing OM.

in various states of preservation (Fig. 1), these samples were treated with two additional initial cleaning steps. For clay removal, otoliths were soaked in 2% sodium polyphosphate and sonicated for 5 min in an ultrasonication bath. Next, otoliths were soaked in bicarbonate-buffered dithionite citrate (pH ~ 7.5, 1 h at 80 °C water bath) as a reductive cleaning agent to remove metals (Mehra and Jackson, 1958). This was followed by an oxidative cleaning with sodium hypochlorite for removal of external OM (Wang et al., 2014), as per modern otoliths (Section 2.1). This external cleaning resulted in fossil otoliths that were devoid of discoloration and sediment (Fig. A2). After external cleaning, the otoliths were snapped to yield half of an intact otolith (Hu and Todd, 1981) for subsequent comparison of otolith halves, as opposed to comparison of differently-broken otoliths. In breaking the otolith, ages were also discernible: Otolith A was larger, from a fish ≥ 9 years old (outer-most growth bands were obscured and indistinguishable), and Otolith B was smaller, from a 3-year-old fish (all growth bands were discernible). The sizes of otolith A and B are similar to those of otoliths in Fig. 1c and d. Otolith halves from each fish were crushed and homogenized with a mortar and pestle and split into two groups per otolith: “Surface and grain cleaning” and “Surface cleaning only” ($n = 3\text{--}4$ replicate subsamples per treatment for each otolith) (Fig. 2). Surface and grain cleaning otolith powder subsamples were weighed to 4.5–5 mg (± 0.01 mg) and treated as above (Sections 2.2–2.4) with sodium hypochlorite cleaning of otolith grains for 12 h followed by DIW rinses, dissolution, oxidation, and $\delta^{15}\text{N}$ analysis. Surface cleaning only powder was not treated with sodium hypochlorite at all after crushing and instead subsamples were weighed to 4.5–5 mg (± 0.01 mg), directly dissolved and oxidized, and then analyzed by the usual protocol as above (Sections 2.3–2.4).

4. TESTS OF ECOLOGICAL APPLICATIONS

4.1. Isotopic identity of wild and farm-raised salmonids

A pilot study was conducted to determine the variability of $\delta^{15}\text{N}_{\text{oto}}$ in farm-raised and wild-caught fish populations, between left and right otoliths from the same individuals, and between intact versus crushed otoliths (Table 2). Otoliths were dissected from farm-raised brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) provided by Musky Trout Hatchery, Asbury, NJ on 24 July 2014. For comparison, otoliths of wild pink salmon (*Oncorhynchus gorbuscha*) were provided by ADF&G, Cordova, Alaska, from fish harvested in the commercial fishery. These fish were caught on 22 August 2008 from two ADF&G management districts (Statistical Areas 222 and 226; see Fig. A3 for map of statistical areas) separated by approximately 40 miles in Prince William Sound, Alaska.

Otoliths of thirteen brown trout and ten rainbow trout were analyzed. Five pairs of pink salmon otoliths from each of two ADF&G districts (a total of ten individuals) were analyzed. For all three species, left (L) and right (R) otoliths from the same fish were compared to test whether $\delta^{15}\text{N}_{\text{oto}}$ varies between sagittal otoliths from the same fish.

Additionally, the R otolith from each of three pink salmon pairs was crushed to compare the $\delta^{15}\text{N}_{\text{oto}}$ of crushed otolith material with that of an intact (uncrushed) otolith that otherwise underwent the same cleaning. Lastly, for both farmed- and wild-caught fish, the relationship between fish size and $\delta^{15}\text{N}_{\text{oto}}$ was investigated.

4.2. Nitrogen isotopic composition of Atlantic cod white muscle tissue

For comparison with $\delta^{15}\text{N}_{\text{oto}}$, approximately 1 cm³ of white muscle tissue per fish was freeze dried for 24 h, then crushed and homogenized with a mortar and pestle, and finally weighed to 1 ± 0.2 mg into tin capsules. The $\delta^{15}\text{N}$ of white muscle tissue ($\delta^{15}\text{N}_{\text{wmt}}$) was determined for each sample using an Isoprime 100TM isotope ratio mass spectrometer interfaced in continuous flow with an elemental analyzer (Vario ISOTOPE cubeTM, Elementar) at Princeton University. The average standard deviation of replicate subsamples was 0.04‰.

4.3. Comparison of historical and modern Atlantic cod

$\delta^{15}\text{N}_{\text{oto}}$ was compared among Atlantic cod from four sources (see also Table 1): (1) fossil otoliths of commercially caught cod from midden deposits of Smuttynose Island, Maine (described above), dated to the 17th century no earlier than 1630 using Lewis Binford Analysis (Binford, 1962; Robinson, 2012); (2) modern cod, landed in Chatham, MA, from a Gulf of Maine sector fishing boat, on November 13th, 2014, and obtained from a fish market (Nassau Seafood, Princeton, NJ); (3) otoliths of two-year-old modern cod collected by the NOAA Fisheries Northeast Fisheries Science Center (NEFSC) from Georges Bank, USA, during fisheries-independent research surveys of the NEFSC Fishery Biology Program in 1981, 1984, 1987, and 2013 between September and November of each year, and (4) lastly, five-year-old farmed cod that had lived their entire post-larval lives in a controlled aquaculture setting at the University of Maine Center for Cooperative Aquaculture Research (CCAR), where the cod were fed commercially formulated aquafeed (Skretting Europa). Only unbroken otoliths were used. Historical otoliths were treated with two additional initial cleaning steps as described above (see Section 3.4 above).

A regression based on otolith weight was used to back-calculate historical fish size. The regression was derived from 463 cod otoliths collected by the Massachusetts Division of Marine Fisheries during the Industry-Based Survey for Gulf of Maine cod, between 2003 and 2007, caught in the western Gulf of Maine between 41.5°N and 44.8°N and in depths less than 138 m (data provided by William Hoffman and Micah Dean, Massachusetts Division of Marine Fisheries).

5. RESULTS

5.1. Reproducibility and precision

Applying the standard protocol, the inter-batch precision (1σ) was 0.3‰ for $\delta^{15}\text{N}_{\text{oto}}$ of two different in-house

standards, respectively made from homogenized otolith powder from Atlantic cod (CDS) and pink salmon (PSS), across eleven sample batches (Fig. 3; Suppl. Table A1). From the cleaning tests, the effects of four factors are summarized below. All results are reported as the mean $\pm 1\sigma$ unless otherwise noted, and p -values are considered significant when below 0.05. All statistical tests and p -values refer to Welch's t -test unless otherwise specified.

- (1) Duration of exposure to the cleaning reagent: Higher temperature facilitated a faster removal of exposed organic matter. The minimum time required for exposure to sodium hypochlorite was approximately six hours for unheated sodium hypochlorite and one hour for heated (60 °C) sodium hypochlorite (Fig. 4). $\delta^{15}\text{N}_{\text{oto}}$ did not significantly differ between short (0.5–1 h) and longer (6–48 h) cleaning duration, although the variance (standard deviation) improved after N content stabilized at 6 h ($7.1 \pm 0.6\text{‰}$ at 0.5–1 h to $7.2 \pm 0.3\text{‰}$ at ≥ 6 h, $p = 0.74$, for room temperature; $7.2 \pm 0.3\text{‰}$ to $6.9 \pm 0.2\text{‰}$ at ≥ 6 h, $p = 0.10$, for 60 °C). N content of uncleaned (0.5 h) otolith powder was 62% higher than otolith powder cleaned for ≥ 6 h (25.2 ± 6.5 nmols N mg^{-1} uncleaned, 15.5 ± 1.0 nmols N mg^{-1} after cleaning; $p = 0.12$).
- (2) Heating *vs.* room temperature cleaning: For room temperature *versus* 60 °C, $\delta^{15}\text{N}_{\text{oto}}$ was not statistically distinct, including samples cleaned for 6 h or more, the time after which N content stabilized for both heated and room temperature treatments ($6.9 \pm 0.2\text{‰}$ heated, $7.2 \pm 0.3\text{‰}$ unheated; Welch's t -test, $p = 0.08$). N content (15.6 ± 0.9 nmols N mg^{-1} heated, 15.5 ± 1.0 nmols N mg^{-1} unheated; Welch's t -test, $p = 0.36$) was also not significantly different between the two temperature treatments.

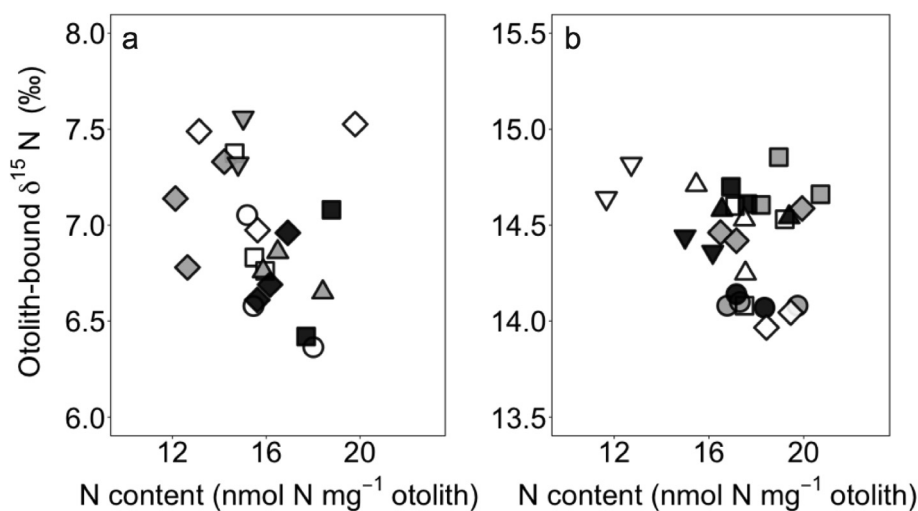


Fig. 3. Analytical precision of the method for in-house standards made of otoliths from two species, Atlantic cod (a) and pink salmon (b). Each symbol type corresponds to a different analysis batch. The inter-batch mean for cod otolith standard (CDS) is $6.9 \pm 0.3\text{‰}$ and the N content is 15.8 ± 1.8 nmols N mg^{-1} (11% percent standard deviation) across eight analysis batches. Pink salmon otolith standard (PSS) is $14.4 \pm 0.3\text{‰}$ and the N content is 17.1 ± 2.0 nmols N mg^{-1} (12% percent standard deviation) across eleven analysis batches.

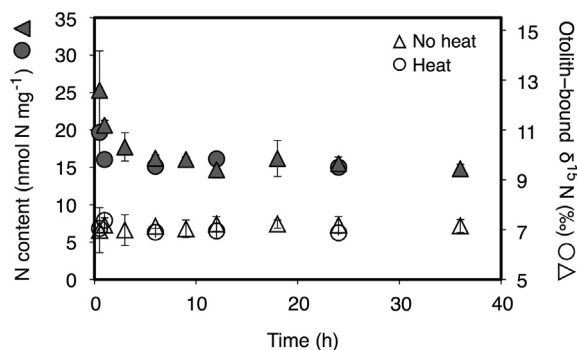


Fig. 4. Time course of cod otolith standard (CDS) exposure to sodium hypochlorite. Samples were either heated using a water bath (60 °C) or maintained at room temperature (22 °C). Despite declining N content over the first 6 h, $\delta^{15}\text{N}_{\text{oto}}$ was similar across all time points within each treatment. N content was not significantly different between the two treatments (15.6 ± 0.9 nmols N mg^{-1} heated, 15.5 ± 1.0 nmols N mg^{-1} unheated; Welch's t -test, $p = 0.36$).

- (3) Grain size: Grain size had no significant effect on $\delta^{15}\text{N}_{\text{oto}}$ ($p > 0.40$ for all grain sizes) for either POR- or sodium hypochlorite-cleaned CDS (Fig. 5). However, $\delta^{15}\text{N}_{\text{oto}}$ standard deviation was highest for the largest grain sizes (1.2 and 0.7‰ for POR- and sodium hypochlorite-cleaned 425 μm grain sizes, compared to 0.1–0.5‰ for all other grain sizes and cleaning treatments). For N content, grain size had no statistically significant effect ($p > 0.20$ in all cases). As a minor exception, aggregating across both POR and sodium hypochlorite cleaning treatments, the 38–150 μm grain size was significantly lower in N content than 150–425 μm grain size (mean N content was

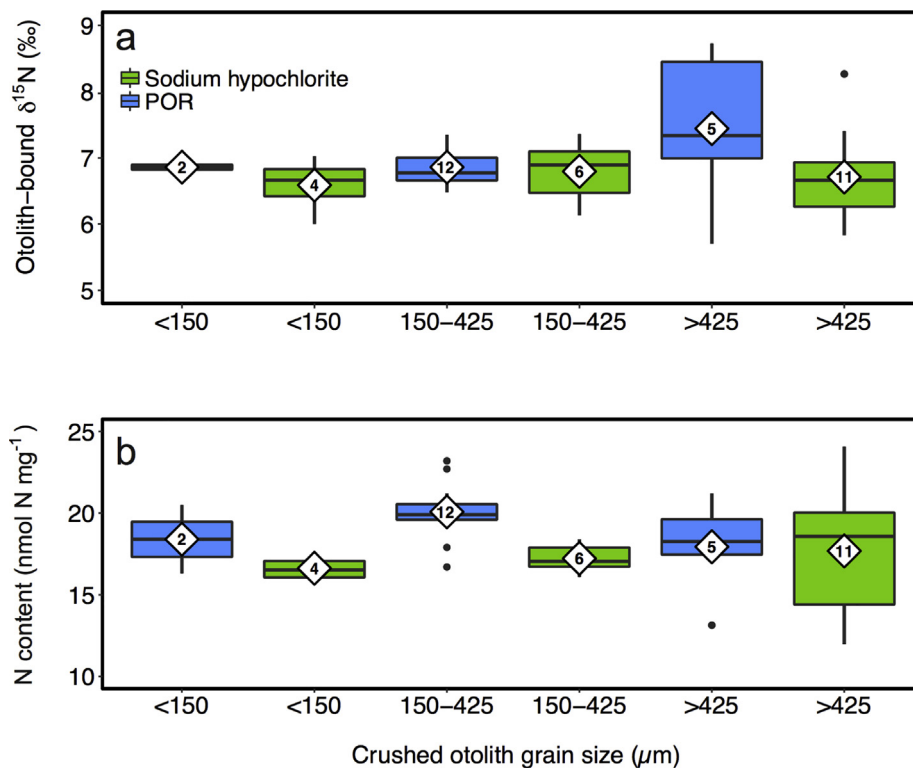


Fig. 5. Effect of grain size and cleaning reagent on of $\delta^{15}\text{N}_{\text{oto}}$ and N content. Boxplots showing interquartile range of $\delta^{15}\text{N}_{\text{oto}}$ (a) and N content (b) of cod otolith standard for three grain sizes and two cleaning reagents. Outliers are plotted as black symbols. The sample mean for each group is indicated by the diamond symbol, with the n for each group inside the symbol. Despite higher variability of both $\delta^{15}\text{N}_{\text{oto}}$ and N content at larger grain sizes, the $\delta^{15}\text{N}_{\text{oto}}$ and N content are not statistically different across any combination of grain sizes ($p > 0.20$ in all cases) other than mean N content for <150 μm vs. 150–425 μm when aggregating across both cleaning treatments (mean N content was 19.1 ± 2.0 nmols N mg^{-1} for 150–425 μm , 17.2 ± 1.7 nmols N mg^{-1} for <150 μm ; $p = 0.05$).

19.1 ± 2.0 nmols N mg^{-1} for 150–425 μm grain size, 17.2 ± 1.7 nmols N mg^{-1} for 38–150 μm grain size; $p = 0.05$).

- (4) Cleaning reagents: POR and sodium hypochlorite cleaning of the 150–425 μm grain sizes of CDS, PSS, and QSN were investigated. There was no significant difference in $\delta^{15}\text{N}_{\text{oto}}$ between POR- and sodium hypochlorite-cleaned CDS (Table 3). However, for PSS and QSN, POR cleaning resulted in lower $\delta^{15}\text{N}_{\text{oto}}$ by 0.8‰ and 0.4‰ respectively. N content was 10% higher for POR-treated CDS compared to sodium hypochlorite-treated samples. For PSS and QSN, N content of POR-treated samples was higher

by 47% and by 342% respectively compared to sodium hypochlorite-treated samples (Table 3). These starkly higher N contents for cleaning by POR and sodium hypochlorite very likely derive from aragonite dissolution and calcite reprecipitation at the high temperature of POR cleaning (Section 6.2).

5.2. Historical otolith testing

For Otolith A, both $\delta^{15}\text{N}_{\text{oto}}$ and N content decreased significantly after cleaning ($p < 0.05$ for both $\delta^{15}\text{N}_{\text{oto}}$ and

Table 3
Effects of cleaning reagent on three species.*

		NaOCl	n	POR	n	P
$\delta^{15}\text{N}$ (‰ vs. air)	CDS	6.80 ± 0.47	6	6.86 ± 0.27	12	0.77
	QSN	15.21 ± 0.31	20	14.90 ± 0.05	2	<0.05
	PSS	14.40 ± 0.30	6	13.61 ± 0.33	8	<0.001
N content (nmol N mg^{-1})	CDS	17.2 ± 0.9	6	20.1 ± 1.8	12	<0.05
	QSN	34.5 ± 5.0	20	50.6 ± 2.8	2	<0.05
	PSS	19.2 ± 2.0	6	84.8 ± 22.3	8	<0.001

* Statistical significance was conducted with a Welch's t -test.

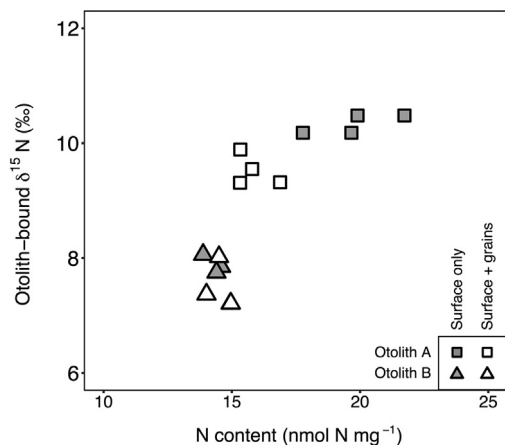


Fig. 6. Effect of cleaning on fossil otoliths. “Surface only” (filled symbols) refers to cleaning of the whole, intact otolith, prior to crushing, and “Surface and grains” (open symbols) refers to cleaning of the crushed otolith grains in addition to cleaning of the intact otolith. Otolith A (square symbols) was a larger otolith and Otolith B (triangle symbols) was a smaller otolith.

N content), but no significant change in $\delta^{15}\text{N}_{\text{oto}}$ or N content was observed for Otolith B ($p = 0.11$ for $\delta^{15}\text{N}_{\text{oto}}$, $p = 0.35$ for N content). With cleaning, $\delta^{15}\text{N}_{\text{oto}}$ changed from $10.3 \pm 0.2\text{‰}$ to $9.5 \pm 0.3\text{‰}$ for fossil Otolith A and from $7.9 \pm 0.2\text{‰}$ to $7.5 \pm 0.4\text{‰}$ for fossil Otolith B (Fig. 6). The N content decreased from 19.8 ± 1.6 to 15.8 ± 0.7 nmol N mg^{-1} after cleaning for Otolith A (i.e., uncleaned powder had 25% higher N content than cleaned powder for Otolith A), but showed no difference for Otolith B, in which the uncleaned powder was 14.3 ± 0.3 and the cleaned powder was 14.6 ± 0.5 nmol N mg^{-1} . The crushing without subsequent cleaning performed in this test could introduce contaminant N. However, Otolith B has indistinguishable N content between uncleaned and cleaned treatments, and the four uncleaned subsamples from Otolith A are uniformly higher in N content relative to cleaned subsamples. Therefore, the differences in N content and $\delta^{15}\text{N}_{\text{oto}}$ between Otolith A and Otolith B are best interpreted in terms of otolith-associated organic matter rather than contamination during grinding.

5.3. Otolith-bound organic matter in wild and farm-raised fish

For the farm-reared brown trout and rainbow trout, which were fed the same food, average $\delta^{15}\text{N}_{\text{oto}}$ was $11.5 \pm 0.3\text{‰}$ ($n = 13$) and $11.3 \pm 0.4\text{‰}$ ($n = 10$), respectively (Fig. 7), and $\delta^{15}\text{N}_{\text{oto}}$ of the two species were not significantly different ($p = 0.28$). Average N content in brown trout was greater than in rainbow trout (21.8 ± 2.4 nmol N mg^{-1} vs. 18.1 ± 3.9 nmol N mg^{-1} , $p < 0.05$; Suppl. Table A1).

Average $\delta^{15}\text{N}_{\text{oto}}$ for wild pink salmon was $14.5 \pm 0.4\text{‰}$ (Fig. 7). The average for crushed otoliths was $14.4 \pm 0.6\text{‰}$ ($n = 3$) and for intact otoliths was $14.5 \pm 0.4\text{‰}$ ($n = 16$). The average $\delta^{15}\text{N}_{\text{oto}}$ difference between crushed and intact otoliths from the same fish was $-0.2 \pm 0.3\text{‰}$ (crushed-

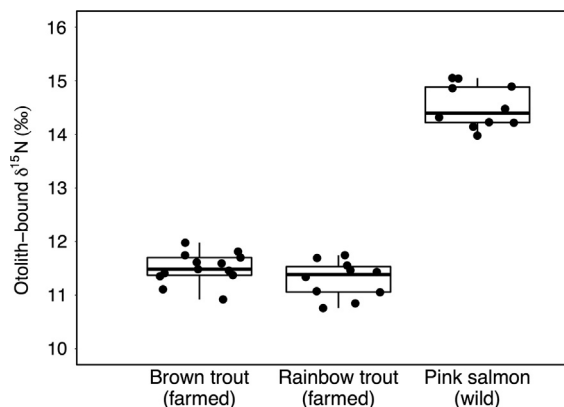


Fig. 7. Cohort-level variability in $\delta^{15}\text{N}_{\text{oto}}$. Intra-cohort variability of farmed brown trout, farmed rainbow trout, and wild pink salmon ($11.5 \pm 0.3\text{‰}$, $11.3 \pm 0.4\text{‰}$, $14.5 \pm 0.4\text{‰}$ respectively). Boxplots show the interquartile range; individual datapoints for all fish individuals are also plotted. Brown trout and rainbow trout were reared on the same formulated commercial feed. Pink salmon were commercially harvested from the wild fishery in Prince William Sound, Alaska and are from the same cohort as opposed to a diverse population of mixed-age individuals.

intact; Fig. 8; $n = 3$). Statistical Area 222 was not significantly different than Area 226 ($p = 0.85$). For N content, crushed pink salmon otoliths yielded an average N content of 17.1 ± 2.0 nmol N mg^{-1} whereas intact otoliths contained 32.0 ± 3.2 nmol N mg^{-1} , or 87% more N than crushed otoliths after cleaning.

Otolith weight for whole pink salmon otoliths ranged from 2.5 to 4.2 mg, averaging 3.3 mg (Fig. 9). Despite this small mass analyzed, the average standard deviation of L and R otoliths from the same fish was only 0.2‰ (Fig. 8).

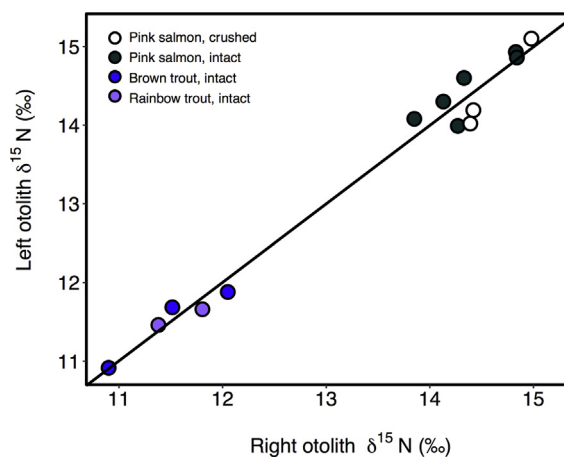


Fig. 8. Left versus right $\delta^{15}\text{N}_{\text{oto}}$. Left (L) versus right (R) otolith for brown trout (blue symbols), rainbow trout (purple symbols), and pink salmon (gray and empty symbols) showing greater variability among individual fish than between L vs. R otoliths from the same fish. Intact otolith (empty symbol) versus crushed otolith (filled symbol) otolith analysis did not affect $\delta^{15}\text{N}_{\text{oto}}$. L vs. R otoliths were correlated ($R^2 = 0.98$, $p < 0.001$).

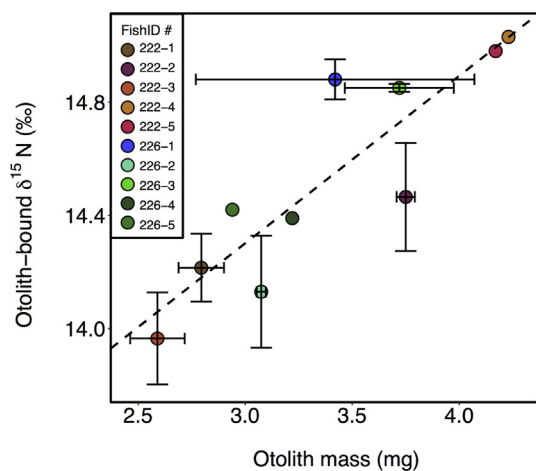


Fig. 9. Otolith mass vs. $\delta^{15}\text{N}_{\text{oto}}$ for wild pink salmon. Otolith mass (a proxy for fish size) versus $\delta^{15}\text{N}_{\text{oto}}$ for individual wild pink salmon showing a positive relationship between fish size and fish $\delta^{15}\text{N}_{\text{oto}}$ of intact otoliths even within a small range of $\delta^{15}\text{N}$ (Pearson correlation, $r = 0.88$, $p < 0.001$). Error bars for otolith mass are 1σ of left and right otoliths from the same fish (otoliths without error bars are only intact otoliths; the otoliths that were crushed prior to analysis were not weighed). Warmer colors are fish from Statistical Area 222; cooler colors are from statistical Area 226 in Prince William Sound, Alaska. This demonstrates that $\delta^{15}\text{N}_{\text{oto}}$ shows a higher trophic level of bigger fish, consistent with the idea that larger fish are able to consume larger prey and thus reside at a higher trophic level relative to conspecifics. The smallest otolith measured was 2.5 mg.

In general, when combining across all three salmonid species (Fig. 8), L vs. R otoliths were not significantly different ($p = 0.998$) and were highly correlated (Pearson correlation, $r = 0.99$, $p < 0.001$). Focusing on the pink salmon alone, L vs. R otoliths were not statistically different (Fig. A4; $p = 0.82$) and were again highly correlated (Pearson correlation, $r = 0.83$, $p < 0.001$). There was not a significant correlation between otolith size and otolith N content (Pearson correlation, $r = -0.35$, $p = 0.35$). The relationship between otolith size and $\delta^{15}\text{N}_{\text{oto}}$ for wild pink salmon was significant (Fig. 6; Pearson correlation, $r = 0.88$, $p < 0.001$) even within a very small size range, as would be expected given that otolith size is correlated with fish size and that fish size is correlated with trophic level for this species (Aydin et al., 2005). In contrast, there was no relationship between otolith size and $\delta^{15}\text{N}_{\text{oto}}$ for farm-reared fish (Fig. A5; Pearson correlation, $r = 0.04$, $p = 0.87$), consistent with the lack of potential for trophic level change or baseline change with increasing size in the fish farm setting.

5.4. Comparison of $\delta^{15}\text{N}_{\text{wmt}}$ to $\delta^{15}\text{N}_{\text{oto}}$

$\delta^{15}\text{N}_{\text{wmt}}$ averaged $15.1 \pm 0.7\text{‰}$ for modern cod and $12.8 \pm 0.5\text{‰}$ for farmed cod. $\delta^{15}\text{N}_{\text{wmt}}$ and $\delta^{15}\text{N}_{\text{oto}}$ from the same fish were linearly correlated (Fig. 10; Pearson correlation, $r = 0.80$, $p < 0.001$) with a slope of $0.69 (\pm 0.33$ 95% confidence interval) and intercept of $-2.7 (\pm 4.78$ 95% confidence interval). $\delta^{15}\text{N}_{\text{wmt}}$ was on average $7.3 \pm 0.7\text{‰}$ higher than $\delta^{15}\text{N}_{\text{oto}}$ from the same fish individual, and there was no significant difference in the $\delta^{15}\text{N}_{\text{wmt}}$ to $\delta^{15}\text{N}_{\text{oto}}$ offset

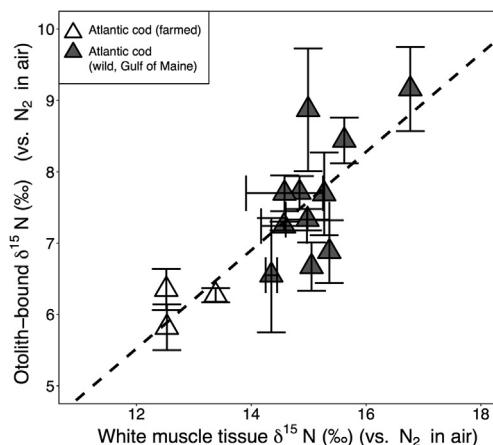


Fig. 10. Paired measurements of $\delta^{15}\text{N}_{\text{wmt}}$ vs. $\delta^{15}\text{N}_{\text{oto}}$ for Atlantic cod. Paired otolith and white muscle $\delta^{15}\text{N}$ for farmed (open triangles) and wild (filled triangles) Atlantic cod. Dashed line shows the least squares regression between $\delta^{15}\text{N}_{\text{wmt}}$ and $\delta^{15}\text{N}_{\text{oto}}$ ($y = 0.69x - 2.74$; $r^2 = 0.64$). Y-error bars denote one standard deviation of replicate otolith subsamples. X-errors bars denote one standard deviation of replicate white muscle measurements if duplicate samples were measured.

for wild versus farmed cod ($7.5 \pm 0.7\text{‰}$ offset compared to $6.7 \pm 0.5\text{‰}$ offset; $p = 0.07$). Based on the above regression, average $\delta^{15}\text{N}_{\text{wmt}}$ was predicted to be 18.6‰ from fossil otoliths.

5.5. Comparison of 17th and 21st century Atlantic cod

Historical Atlantic cod $\delta^{15}\text{N}_{\text{oto}}$ averaged $10.0 \pm 0.6\text{‰}$ (ranging from 9.6 to 10.7‰), while modern commercially harvested cod $\delta^{15}\text{N}_{\text{oto}}$ averaged $7.9 \pm 0.9\text{‰}$ (ranging from 6.7 to 9.2‰) and the two groups were significantly different (Fig. 11, Fig. A6; $p < 0.001$). Wild age-2 Georges Bank (GB) cod from NEFSC autumn research survey were $8.1 \pm 0.7\text{‰}$ (ranging from 6.9 to 9.8‰) which was indistinguishable from commercially harvested Gulf of Maine (GOM) cod ($p = 0.52$) but significantly different from fossil cod $\delta^{15}\text{N}_{\text{oto}}$ ($p < 0.001$). For farm-raised cod, the average $\delta^{15}\text{N}_{\text{oto}}$ was $6.2 \pm 0.3\text{‰}$ (ranging from 5.8 to 6.4‰). Using the $\delta^{15}\text{N}_{\text{wmt}}$ to $\delta^{15}\text{N}_{\text{oto}}$ linear regression above (Section 5.4), predicted $\delta^{15}\text{N}_{\text{wmt}}$ of historical cod may have ranged from 18.0 – 19.5‰ . The N content was 14.6 ± 1.1 nmol N mg^{-1} and 16.1 ± 1.6 nmol N mg^{-1} for historical and modern GOM cod, respectively, and they were not significantly different ($p = 0.11$). GB cod were 16.3 ± 1.6 nmols N mg^{-1} and were not significantly different from modern GOM cod ($p = 0.84$) or fossil GOM cod ($p = 0.06$). For farmed cod, N content was 21.3 ± 1.7 nmol N mg^{-1} . Since the farm-raised cod had a food source unrelated to that available in the Gulf of Maine, only the N content results are relevant for comparison to the wild cod. The average otolith mass for historical, wild caught, and farmed cod was 791.68 ± 254.03 mg, 533.55 ± 86.71 mg, and 480.81 ± 21.19 mg. If converted to fish size, based on a relationship of otolith mass to fish length from modern fish (Fig. A7;

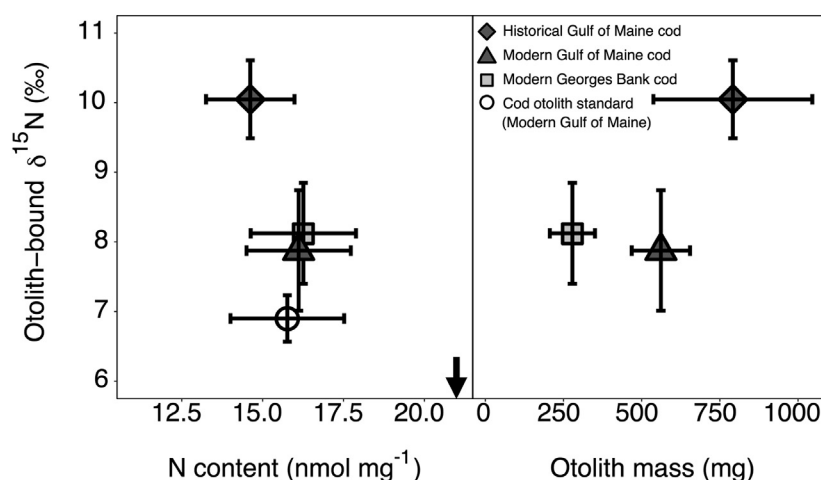


Fig. 11. $\delta^{15}\text{N}_{\text{oto}}$ and N content for historical and modern Atlantic cod. $\delta^{15}\text{N}_{\text{oto}}$, N content, and otolith mass mean $\pm 1\sigma$ for 17th century Atlantic cod otoliths from a historical fishing station in the western Gulf of Maine (diamond symbol; $n = 4$ fish), for modern commercially-harvested Atlantic cod from the western Gulf of Maine (triangle symbol; $n = 7$ fish), and for modern Georges Bank cod (square symbol; $n = 18$ fish). The inter-batch mean for CDS is also plotted (circle symbol; $n = 19$ measurements from homogenized CDS made of four additional modern Gulf of Maine cod otoliths). The N content for farm-raised Atlantic cod (black arrow; $n = 3$ fish) is plotted without $\delta^{15}\text{N}_{\text{oto}}$ because fish were reared on commercial aquafeed not relevant for the comparison to wild fish.

$n = 463$), we find that this translates to a range of 64–97 cm (mean 84 ± 15 cm) for historical cod, and a range of 58–78 cm (mean 70 ± 7 cm) for wild modern cod.

6. DISCUSSION

Key aspects of the new isotope method are discussed first, then the ecological signals recorded in otoliths are discussed. The sections that follow proceed from the lab bench to environmental samples, and lastly to preliminary historical application of $\delta^{15}\text{N}_{\text{oto}}$ as an investigation of the validity of this approach for fossil otoliths.

6.1. Effects of oxidative cleaning on otolith powders from modern and fossil otoliths

6.1.1. Modern otoliths

The organic fraction of otoliths, often called the “organic matrix”, is composed of insoluble, collagen-like proteins and soluble, high molecular weight organic molecules that together are thought to control the structure and morphology of the mineral fraction (Degens et al., 1969; Söllner et al., 2003; Falini et al., 2005), as in other biogenic carbonates (Weiner, 1984; Belcher et al., 1996). The configuration of the organic matrix and its interaction with the mineral fraction in otoliths and other biominerals is an active field of study (DeVol et al., 2015; Wojtas et al., 2015; Mao et al., 2016). Organic matter (OM) concentration varies at multiple spatial scales within the otolith, from nanometers to millimeters (Dunkelberger and Dean, 1980; Watabe et al., 1982; Morales-Nin, 1986; Dauphin and Dufour, 2008). The molecular-level associations are likely diverse, probably covering a spectrum between truly

intracrystalline and intercrystalline, and are beyond the scope of this study. Here, “protected” OM refers to OM that is sufficiently trapped within the crushed otolith grains that it can only be accessed once the mineral fraction is dissolved. In contrast, “exposed” OM is OM that is removed by treatment with a harsh oxidant dissolved in water (Fig. 2). Accordingly, the distinction between protected and exposed OM will depend, for example, on whether the otolith was ground prior to cleaning.

In the present study, we evaluated the scale at which otolith OM is accessible to a harsh oxidant by analyzing crushed otolith powders of grain sizes between 60 μm and 425 μm . We found that otolith N content and $\delta^{15}\text{N}$ were unchanged regardless of surface-area-to-volume exposed to the sodium hypochlorite prior to crushing and that the standard deviation of replicate subsamples was not affected by grain size, except for the largest grain sizes, which had higher standard deviations likely due to the lower number of grains per sample. The fact that the cleaning methods are efficient across all grain sizes implies that the OM composition is broadly consistent across the otolith. At the same time, smaller grain sizes increased the precision, which suggests heterogeneity at some scales within the otolith; as otoliths record the entire life history of fishes, some heterogeneity over fish lifetime is expected.

One concern is that extended oxidative cleaning might remove a significant fraction of the otolith-native OM. We found that the operationally defined protected OM remains so across a time course of exposure to sodium hypochlorite (Fig. 4). N content stabilized after only six hours, without evidence of further decline out to 36 h. This suggests that otolith grains are not porous on a scale that allows the cleaning methods to continuously access

otolith-native OM. Additionally, sodium hypochlorite cleaning at 60 °C yielded a final N content that was indistinguishable from sodium hypochlorite cleaning at room temperature, although the heating treatment resulted in faster removal of exposed N, stabilizing after 1 h rather than 6 h. This is consistent with heated versus room temperature cleaning of ostracod valves (Bright and Kaufman, 2011) and ratite eggshell (Brooks et al., 1990; Crisp et al., 2013), suggesting the existence of a pool of well-protected OM in at least some biogenic minerals.

Uncleaned cod otolith grains (CDS) have up to 62% more N compared to cleaned otolith grains (Fig. 4). Intact pink salmon otoliths contain 87% more N than crushed otoliths. Cleaning removes this additional N, leaving only the operationally defined protected OM for subsequent analysis. The results of the time tests and of the intact vs. crushed otolith experiment for pink salmon show that exposed and protected OM have indistinguishable $\delta^{15}\text{N}$ (Figs. 2 and 3). This argues that, in non-fossil otoliths, the two OM classifications are not fundamentally different from one another other than in the degree of protection afforded by the mineral. More broadly, the overall protected nature of the otolith-bound OM is consistent with studies on coral (Ingalls et al., 2003; Wang et al., 2014, 2015), foraminifera (Ren et al., 2009; Straub et al., 2013), and clam shell (Crenshaw, 1972), wherein OM remains protected during continued exposure to harsh oxidative cleaning.

6.1.2. Fossil otoliths

The efficacy of oxidative cleaning must also be assessed for fossil otoliths from diagenetically active environments (Fig. 6). Diagenetic processes can alter the $\delta^{15}\text{N}$ of OM, impeding interpretation in terms of primary biological or environmental processes. Thus, diagenetically exposed OM, which may have been altered or contaminated by diagenesis, should be removed prior to isotope analysis. Diagenetic N loss most often elevates $\delta^{15}\text{N}$ (Robinson et al., 2012, and references therein). Under typical open ocean conditions of low organic matter preservation and oxic bottom waters, OM buried in marine sediments is higher than the $\delta^{15}\text{N}$ of the OM delivered to the seabed (Altabet and Francois, 1994; Altabet, 2006). Similarly, the $\delta^{15}\text{N}$ of suspended OM in deep ocean waters is $\geq 3\text{‰}$ higher than the OM sinking into the ocean interior (Saino and Hattori, 1980; Altabet et al., 1991; Casciotti et al., 2008; Hannides et al., 2013). Evidence for preferential diagenetic loss of OM with low $\delta^{15}\text{N}$ also comes from studies of relict organic-rich layers in deep sea sediments (Möbius et al., 2010) as well as studies of soils (Natelhoffer and Fry, 1988), peat bogs (Macko et al., 1990), and salt marshes (Fogel et al., 1989).

The degree of isotopic alteration by diagenesis appears to depend on conditions. Under the high OM preservation and low-oxygen conditions of certain isolated marine basins and productive margin settings, smaller differences are observed between sinking and buried OM $\delta^{15}\text{N}$ (Altabet et al., 1999; Ganeshram et al., 2000; Thunell et al., 2004; Robinson et al., 2012). Studies of buried Mediterranean sapropels (Möbius et al., 2010) and buried *Spartina* marsh

grasses (Tremblay and Benner, 2006) also suggest that high preservation and/or anoxic conditions can prevent a clear rise in $\delta^{15}\text{N}$ with diagenesis. In contrast, substantial elevation of ($\geq 3\text{‰}$) is frequently observed in open ocean settings (Altabet and Francois, 1994). In parallel, laboratory studies suggest variation in the isotopic impact of diagenesis with redox condition (Lehmann et al., 2002). Moreover, externally sourced N can also be added to sedimentary materials during deposition, burial, and diagenesis, further overprinting the primary isotopic signal (e.g., Schubert and Calvert, 2001; Meckler et al., 2008, 2011; Ren et al., 2009). Given these complexities, the only robust way to address it is to remove OM that may have been exposed to diagenesis.

In the present study, the testing of broken fossil otoliths from the Smuttynose Island midden revealed an apparent difference between cleaned vs. uncleaned otolith powder for one (Otolith A) of the two otoliths (Fig. 6). It must be noted that both Otolith A and B were cleaned externally prior to this analysis (Section 3.4; Fig. 2) and that “cleaning” here refers to cleaned vs. uncleaned crushed otolith powder from otoliths that had already been externally cleaned with sodium polyphosphate, reductive agents, and sodium hypochlorite. The higher $\delta^{15}\text{N}_{\text{oto}}$ and N content of uncleaned material is consistent with the tendency of bacterial diagenesis to cause the preferential loss of OM with a lower $\delta^{15}\text{N}$ (Macko et al., 1986; Lehmann et al., 2002). As with modern otoliths, N content is higher in uncleaned fossil otolith powder than in cleaned powder. However, the uncleaned fossil powder had 25% higher N content, whereas uncleaned modern otolith powder had 62% higher N content. The lower starting N content for fossil powder underlines the importance of oxidative cleaning for fossil samples to avoid variability introduced by diagenetic N addition or loss.

N content did not vary significantly as a function of cleaning for Otolith B. Otolith B (similar to Fig. 1d) was missing its rostrum (end), potentially exposing a pathway for both diagenetic fluids and our cleaning solutions to access the interior of the otolith. One possible explanation is that the external cleaning (Section 3.4) was able to access the exposed OM through the extant cracks in this broken otolith, resulting in the leaching of all diagenetically altered or contaminated OM prior to the powder oxidative cleaning step. An alternative explanation is that diagenesis had already removed all affected N through the cracked fraction of the otolith (Fig. A8 shows details of a pitted otolith).

The apparent difference in preservation quality of the two fossil otoliths underlines the importance of the cleaning step. Whether lower N content was caused by the external, surficial cleaning of the whole otolith or due to loss processes occurring in sediments *in situ*, differences in preservation status here would have led to differences in $\delta^{15}\text{N}$ if left unaddressed by the sodium polyphosphate, dithionite citrate, and sodium hypochlorite cleaning. The standard protocol is to clean the intact otolith (“surficial cleaning”), followed by crushing the otolith (in order to weigh the otolith if necessary, and to homogenize and subsample the otolith) and cleaning the resulting otolith powder with sodium hypochlorite (“otolith powder cleaning”) (Fig. 2). For both damaged and fully intact fossil otoliths, the standard

$\delta^{15}\text{N}_{\text{oto}}$ protocol for otoliths (with two cleaning steps, one before and one after otolith grinding) appears to effectively remove exposed OM and results in N content that falls within the range expected for modern cod.

6.2. Differing effects of cleaning reagents on otolith stability

The optimal cleaning is one that is harsh enough to remove diagenetically exposed OM but not so harsh as to alter the $\delta^{15}\text{N}$ of the protected OM (Gaffey and Bronnimann, 1993; Penkman et al., 2008; Bright and Kaufman, 2011). Given the long history of persulfate-based oxidation as a strategy for completely oxidizing environmental organic matter and its use at autoclave temperatures (Bronk et al., 2000), we initially assumed that POR treatment would represent a harsher cleaning than that using sodium hypochlorite at room temperature or 60 °C. Based on this logic, if the two cleanings were to yield different N content, we expected lower N content of POR-cleaned powders relative to the sodium hypochlorite cleaning. However, we observed the opposite tendency, with higher and more variable otolith-bound N content using POR, and the difference was dramatic for two of three otolith standards: PSS and QSN (Table 3). We suspect that the N content difference is due to the recrystallization (or dissolution/reprecipitation) of aragonite to calcite at the high temperature (up to 121 °C) of the POR cleaning. Under these conditions, recrystallization of aragonite to calcite has been observed and described in detail (Lécuyer, 1996; Pokroy et al., 2006; Ruiz-Agudo et al., 2014; Staudigel and Swart, 2016). This transition may have resulted in the trapping of otherwise-external OM on or near the surface of the otolith grains, for example, by the development of a new coating of calcite, before that OM could be fully oxidized by the reagent. The minimal effect on CDS N content may indicate that otolith aragonite formed by cod is more stable than aragonite formed by pink salmon and queen snapper.

6.3. Variation in $\delta^{15}\text{N}_{\text{oto}}$ within individual fish

$\delta^{15}\text{N}_{\text{oto}}$ in left and right otoliths of an individual fish differs less than $\delta^{15}\text{N}_{\text{oto}}$ among individuals, even within a small range of $\delta^{15}\text{N}_{\text{oto}}$ (~1‰; Fig. 8). Thus, an individual fish has an isotopic identity that is recorded by both otoliths. This suggests that the individual-to-individual $\delta^{15}\text{N}$ differences of less than 1‰ can be reconstructed with $\delta^{15}\text{N}_{\text{oto}}$ (Fig. 8) and for very small otoliths (2.5–4.2 mg; Fig. 9). More mechanistically, the L versus R comparison demonstrates that at least some forms of physiologically driven variation in $\delta^{15}\text{N}_{\text{oto}}$ are too small to overprint the environment- and ecology-driven variation in the $\delta^{15}\text{N}$ of individual fish in a population. Thus, even within a fairly homogenous population, individual fish retain an isotopic identity.

6.4. Ability to measure small otoliths

The smallest pink salmon otolith measured here was 2.5 mg, which is the smallest otolith for which $\delta^{15}\text{N}_{\text{oto}}$ has been

measured to our knowledge. Relative to previous methods that required much more otolith material for analysis, the ability to measure small otoliths transforms $\delta^{15}\text{N}_{\text{oto}}$ into a tool that can be used for individual fish. Otolith N content in this study ranged from 15.8 nmols mg^{-1} for wild cod to 21.3 nmols mg^{-1} for farmed cod, and the concentration for intact, uncrushed otoliths is higher (32.0 nmols mg^{-1} for intact pink salmon otoliths). With the current N blank of 0.3–1 nmol for $\delta^{15}\text{N}_{\text{oto}}$, and with the goal of minimizing N blank contribution to <5% of overall N, this means that as little as 0.4 mg of material or as little as 0.2 mg of material for the most OM-poor and OM-rich species in this study, respectively, are required for analysis. Given these low sample size requirements, intra-otolith microsampling may be able to resolve $\delta^{15}\text{N}_{\text{oto}}$ information from different time periods of a fish's life. For example, juvenile and adult $\delta^{15}\text{N}$ from the same fish could be compared by measuring the nucleus and the outer edge of the same otolith, an archival record that is not available from tissues.

6.5. Variation in $\delta^{15}\text{N}$ among individual fish

Two factors, baseline (primary producer) $\delta^{15}\text{N}$ and diet (e.g., trophic level), control the $\delta^{15}\text{N}$ of metazoans. Pink salmon from the same cohort have highly similar life history patterns (Bonar et al., 1989) and thus individuals from the same cohort experience a high degree of similarity in baseline $\delta^{15}\text{N}$ over their lifetimes. Thus, in the present study, $\delta^{15}\text{N}_{\text{oto}}$ variations among pink salmon otoliths studied likely reflect differences in diet. Otolith size is a proxy for fish size under most conditions when fish are the same age (Templeman and Squires, 1956; but see Mosegaard et al., 1988; Wright et al., 1990; Barber and Jenkins, 2001 for exceptions relating to decoupling between fish growth and otolith size). Thus, the positive relationship between $\delta^{15}\text{N}_{\text{oto}}$ and otolith size (Fig. 9) is consistent with a correlation between fish size and diet, with larger fish having higher effective trophic level. This was consistent with our expectations, as this species undergoes an ontogenetic dietary shift (Aydin et al., 2005), and larger pink salmon are capable of consuming larger prey (Aydin et al., 2005; Cross et al., 2005). Additionally, quality prey is linked to higher growth rate for pink salmon in the northern Gulf of Alaska and Prince William Sound (Aydin et al., 2005; Cross et al., 2005). The correlation is not driven by fish age: returning pink salmon are two years old in this region (Bonar et al., 1989). For some species, a higher $\delta^{15}\text{N}_{\text{oto}}$ at greater otolith mass may not necessarily correspond to higher trophic level. For example, Choy et al. (2012) used amino acid-specific $\delta^{15}\text{N}$ and found that variability in bulk muscle tissue $\delta^{15}\text{N}$ of lanternfishes and dragonfishes resulted from variation in baseline $\delta^{15}\text{N}$, as opposed to trophic level. However, baseline $\delta^{15}\text{N}$ has no known reason to covary with the size of otolith (or fish) in pink salmon caught in Prince William Sound. Thus, in this case, the higher trophic level of larger fish within the cohort likely explains the higher $\delta^{15}\text{N}_{\text{oto}}$ of larger otoliths.

As otolith growth increases volumetrically, each new layer of aragonite is volumetrically greater than the previous layer (Anderson et al., 1992). Thus, $\delta^{15}\text{N}_{\text{oto}}$ is weighted

toward the $\delta^{15}\text{N}$ of the most recent diet (assuming that N content is constant among consecutive layers, which is true at least for the fish examined here). Thus, $\delta^{15}\text{N}$ in later life is disproportionately important. For pink salmon in the northern Gulf of Alaska, this effect is likely compounded by the fact that the ontogenetic shift to higher trophic level (and thus higher- $\delta^{15}\text{N}$ prey) is also associated with faster growth due to the higher nutritional quality of the high trophic level diet (Aydin et al., 2005).

Similar to pink salmon for which baseline was similar for all individuals in the same cohort, both species of farmed trout were reared in adjacent freshwater raceways, thus controlling for the $\delta^{15}\text{N}$ of their diet. Since farmed trout and wild pink salmon encountered different diet $\delta^{15}\text{N}$, direct comparison of $\delta^{15}\text{N}_{\text{oto}}$ is not ecologically relevant. As farmed brown trout and rainbow trout consume a formulated fish feed for their post-larval diet, as opposed to wild prey, and consumed this food for their entire life history, we hypothesized that differences in otolith (and thus fish) size would not be correlated to changes in $\delta^{15}\text{N}_{\text{oto}}$, and, indeed, we found no relationship between otolith size and $\delta^{15}\text{N}_{\text{oto}}$ for either species (Fig. A5). This observation serves as a negative control to confirm that $\delta^{15}\text{N}_{\text{oto}}$ is a robust recorder of the $\delta^{15}\text{N}$ of a fish's diet.

6.6. Variation in $\delta^{15}\text{N}_{\text{oto}}$ within groups of wild and farmed fish

The standard deviation of $\delta^{15}\text{N}_{\text{oto}}$ was similar for a wild pink salmon cohort and two farm-reared trout cohorts (0.4‰, 0.3‰ and 0.4‰ respectively). It is surprising that the standard deviation for the wild population is not greater than for farmed fish. However, as pink salmon are usually two years old during their return migration and were harvested in the same geographic area, the pink salmon used in this study share a highly similar life history. Other species with more variable life history traits may have greater among-individual variability, e.g., as found for age-2 GB cod ($1\sigma = 0.7\%$; Fig. 11). The variability in $\delta^{15}\text{N}_{\text{oto}}$ within a group of fish must be determined on a case-by-case basis. Nonetheless, the highly-conserved $\delta^{15}\text{N}_{\text{oto}}$ in wild pink salmon demonstrates the cohort-level fidelity of $\delta^{15}\text{N}_{\text{oto}}$ of at least some wild fish. Lastly, the low standard deviation in $\delta^{15}\text{N}_{\text{oto}}$ among farm-reared individuals implies that physiologically-induced variability is not a significant contributor to the signal in otoliths, indicating that $\delta^{15}\text{N}_{\text{oto}}$ of an entire population may be well represented by a relatively small number of $\delta^{15}\text{N}_{\text{oto}}$ measurements.

6.7. Variation in N content between wild and farmed Atlantic cod

In comparing farmed versus wild Atlantic cod in the Gulf of Maine, we found that large differences in N content can exist within a species—otoliths of farmed cod have 50% higher N content than those of wild cod (Fig. 11; Suppl. Table A1). The higher N content of farmed cod may result from differences in growth rate, diet (formulated aqua-feed for the farmed fish), and metabolism. The higher N content did not significantly affect the $\delta^{15}\text{N}_{\text{oto}}$ offset compared to

$\delta^{15}\text{N}_{\text{wmt}}$. Additionally, brown trout and rainbow trout fed the same food had indistinguishable $\delta^{15}\text{N}_{\text{oto}}$ despite the different N contents. This further confirms that diet plays a dominant role in setting $\delta^{15}\text{N}_{\text{oto}}$.

6.8. Comparison of $\delta^{15}\text{N}_{\text{wmt}}$ to $\delta^{15}\text{N}_{\text{oto}}$

In wild and farmed Atlantic cod, as expected, muscle and otolith $\delta^{15}\text{N}$ were correlated (Fig. 10; Pearson correlation, $r = 0.80$). However, two observations must be explained: (1) the slope of less than one for the otolith versus muscle regression, and (2) the much lower $\delta^{15}\text{N}_{\text{oto}}$ compared to $\delta^{15}\text{N}_{\text{wmt}}$. First, fish muscle turns over on timescales of months to years, depending on metabolism and other factors (e.g., Logan et al., 2006; Ankjær et al., 2012; Madigan et al., 2012; Mohan et al., 2016). Thus, white muscle records a shorter, more recent history compared to the otolith, which is continuously accruing new material and records the entire life history of the fish. As cod are known to have a lower trophic level as smaller fish, and some component of $\delta^{15}\text{N}_{\text{oto}}$ is from the fish's early life whereas $\delta^{15}\text{N}_{\text{wmt}}$ records recent $\delta^{15}\text{N}$, we hypothesized that the slope would be <1 . This hypothesis based on differing temporal integration is consistent with data shown in Fig. 10. Second, the low $\delta^{15}\text{N}_{\text{oto}}$ compared to $\delta^{15}\text{N}_{\text{wmt}}$ was first reported by Grønkjær et al. (2013), who also measured Atlantic cod otoliths. Grønkjær et al. (2013) found that cod otolith $\delta^{15}\text{N}$ records the diet directly, without the usual trophic offset found in tissues. One important distinction is that Grønkjær et al., 2013 measure only soluble OM for comparisons with dietary $\delta^{15}\text{N}$ whereas the current study measures bulk OM comprising both soluble and insoluble fractions. As the soluble fraction comprises approximately two-thirds of otolith OM by mass (Grønkjær et al., 2013), the finding that otolith OM was isotopically similar to diet is still relevant for the current study and provides another example of lower $\delta^{15}\text{N}_{\text{oto}}$ compared to $\delta^{15}\text{N}_{\text{wmt}}$ for this species. Moreover, there is no *de facto* reason that otolith $\delta^{15}\text{N}$ and muscle $\delta^{15}\text{N}$ should be identical, as different proteins are likely used in the construction of different fish components, and these vary in amino acid composition. Indeed, fractionation of other tissues (liver, scales, muscle, blood plasma) relative to diet has been shown to be variable, even within the same fish (Macneil et al., 2005; Logan et al., 2006; Buchheister and Latour, 2010). Tissue-specific patterns in nitrogen isotope fractionation are usually attributed to differences in amino acid concentrations and also in the degree of amino acid routing to different tissues (McMahon et al., 2010; Mohan et al., 2016). Regardless of the offset, the high correlation indicates that the factors that control muscle $\delta^{15}\text{N}$ also affect otolith $\delta^{15}\text{N}$. This demonstrates the suitability of $\delta^{15}\text{N}_{\text{oto}}$ for investigating the same types of ecological questions as $\delta^{15}\text{N}_{\text{wmt}}$.

6.9. Comparison of 17th and 21st century Atlantic cod

Fossil $\delta^{15}\text{N}_{\text{oto}}$ is 2.2‰ higher than modern otoliths. Using the otolith versus muscle $\delta^{15}\text{N}$ relationship (Fig. 10), the muscle $\delta^{15}\text{N}$ of the measured 17th century

cod at the end of their lives is predicted to be 3.5‰ higher than modern cod (18.6‰ compared to 15.1‰) from the same region. This is calculated based on the regression $\delta^{15}\text{N}_{\text{oto}} = 0.69 (\pm 0.33 \text{ 95\% confidence interval}) * \delta^{15}\text{N}_{\text{wmt}} - 2.74 (\pm 4.78 \text{ 95\% confidence interval})$. We interpret the $\delta^{15}\text{N}_{\text{wmt}}$ with caution because of the low sample size, and also because no otoliths from similarly large modern fish were available for comparison with the otoliths from ~meter-long cod in the midden. Nevertheless, the finding that fossil cod $\delta^{15}\text{N}_{\text{oto}}$ is 2.2‰ higher than the modern value calls for interpretation.

Two possible causes for change in cod trophic level (and thus $\delta^{15}\text{N}_{\text{oto}}$) can be identified. First, cod trophic level tends to rise with fish size, as fish size in itself changes the prey items that can be consumed by gape-limited predators such as cod. Second, environmental and ecological changes can cause a change in the $\delta^{15}\text{N}$ of the prey available to cod even without a change in the size of the cod (i.e. “trophic-level-at-size”). We address these in turn.

6.9.1. Role of fish size

Cod are known to become increasingly piscivorous (fish-consuming) as they grow, including increasingly cannibalistic, based on stomach content data (Bigelow and Schroeder, 1953; Pålsson, 1983; Link and Garrison, 2002b; Smith et al., 2007; Pålsson and Björnsson, 2011). In the Gulf of Maine, stomach content data spanning 1973–1998 indicate that small cod (31–40 cm) consumed mostly crustaceans (an average of 54% by volume) and a smaller contribution of fish (18%) whereas the diet of large cod (81–90 cm) contained predominantly fish (66%) (Link and Garrison, 2002b). For cod in the largest size range (>120 cm), diet also included significant contributions from bluefish, goosefish, and redfish, in addition to the fish species listed above (for a total of 83% fish) (Link and Garrison, 2002b). The increasing percentage of fish prey, including shifts in species with increasing cod size, suggest that a size-related increase in cod $\delta^{15}\text{N}$ is very likely. Since fish prey usually have higher $\delta^{15}\text{N}$ than macroinvertebrate prey in this region (e.g., Fry, 1988; but see Sherwood and Rose, 2005, for examples in which pelagic and benthic prey can have overlapping $\delta^{15}\text{N}$ due to differing baselines), large cod would be expected to have higher $\delta^{15}\text{N}$ than small cod due to a higher proportion of fish in the diet of large cod. The specific relationship between cod size and $\delta^{15}\text{N}$ has not been investigated in the Gulf of Maine to our knowledge. However, Jennings et al. (2002) find that Atlantic cod in the North Sea increase by 3‰ from 40 to 140 cm. Other piscivorous fish species that are known based on stomach contents to shift from lower trophic level prey to higher trophic level prey undergo a 1–4‰ increase in $\delta^{15}\text{N}$ between intermediate and large lengths (e.g., Hobson and Welch, 1995; Jennings et al., 2002; Graham et al., 2007; Wells et al., 2008; Christiansen et al., 2012; Glaz et al., 2012; Kim et al., 2012; Ramsvatn and Pedersen, 2012; Weng et al., 2015). The $\delta^{15}\text{N}$ of Arctic char, a similarly piscivorous (and cannibalistic) species as cod, increases by 3.7‰ from intermediate lengths to large lengths (i.e., from 30 cm to 50 cm; Hobson and Welch, 1995). In the present study, the largest midden otolith is

estimated to have come from a 97 cm cod whereas the largest modern otolith came from a 78 cm cod. (The finding that historical cod were larger is not surprising, as declining body size of cod in the Gulf of Maine has been well documented; Jackson, 2001; Barot et al., 2004; Bourque et al., 2008; NEFSC, 2012). In this context, it is possible that the 2‰ higher $\delta^{15}\text{N}_{\text{oto}}$ in midden cod is entirely or partly due to the effect of their larger size on their trophic position. To understand the importance of this factor, data are required to assess the effect of size on cod $\delta^{15}\text{N}_{\text{oto}}$ in the Gulf of Maine.

Environmental and ecological change may also have contributed to the apparent change in cod $\delta^{15}\text{N}$. Willis et al. (2013) find that the percentage of fish in cod diet, relative to macroinvertebrates, declined from 70% to 29% from 1965 to 2005 in the Gulf of Maine, an effect that was independent of cod size. A recent comparison of cod with and without access to herring prey found ~1‰ higher $\delta^{15}\text{N}$ -at-size of cod in regions where herring are abundant (Willis et al., 2016). It is thought that the degree of cannibalistic behavior by cod has also declined in the Gulf of Maine region (Tsou and Collie, 2001; Link and Garrison, 2002a,b; Carr and Kaufman, 2009). This decrease has been attributed to both smaller population size (cod population size has declined by an order of magnitude since the late 1800s; Alexander et al., 2009) and also because of fewer large cod that can prey on smaller ones. This cannibalism effect conflates changes in population size and cod size. In summary, a lower modern cod $\delta^{15}\text{N}$ is consistent with changing prey patterns in addition to the changes in cod size. Potential changes in $\delta^{15}\text{N}$ -at-size, which may reflect large scale environmental and ecological change, will be elucidated in future work.

6.9.2. Possible role of baseline $\delta^{15}\text{N}$ change

Possible approaches to address baseline changes include the following: (1) comparing $\delta^{15}\text{N}$ changes in multiple fish species, with the logic that a shared change among species with different prey would most likely reflect a baseline change (2) measuring the shell-bound $\delta^{15}\text{N}$ of primary consumers, e.g. bivalves or foraminifera, or producers, e.g. diatoms, if preserved in the same sediments, and (3) developing methods for compound-specific isotope analysis (CSIA) of $\delta^{15}\text{N}$ for otolith OM, as some amino acids (AAs) appear to record baseline without trophic elevation, while other AAs record trophic level as well (McClelland and Montoya, 2002; Chikaraishi et al., 2009). To date, CSIA is the only method to determine baseline and trophic effects from the same sample. McMahan et al. (2011b) introduced a method by which amino acid-specific $\delta^{13}\text{C}$ in otolith OM was analyzed and applied to retrospectively determine nursery grounds and migratory patterns of snapper in the Red Sea (McMahan et al., 2011b, 2012). However, no comparable methods have been applied to N isotopes, due to the low N content of otoliths. Moreover, beyond the issue of sensitivity, given the existing data on amino acid N isotopes, the approach may not be adequately precise to shed light on the relatively modest (e.g., ~1‰) changes that can currently be identified by bulk $\delta^{15}\text{N}_{\text{oto}}$ analysis. Method

development and ground-truthing may address these issues in the future.

Here, for explanatory purposes, we consider possible influences of baseline $\delta^{15}\text{N}$ change on the observed change in cod $\delta^{15}\text{N}_{\text{oto}}$. The dominant driver of baseline $\delta^{15}\text{N}$ variation in the open ocean is the $\delta^{15}\text{N}$ of nitrate assimilated into biomass in surface waters. $\delta^{15}\text{N}$ of this assimilated nitrate is controlled by both the $\delta^{15}\text{N}$ of the nitrate supply and the degree of nitrate consumption in surface waters. In the Gulf of Maine coastal region, the degree of consumption is usually complete over the course of the spring/summer growth period, which would tend to make the $\delta^{15}\text{N}$ of the nitrate supply the dominant driver of baseline change.

In the case of the North Atlantic there are regional variations in the $\delta^{15}\text{N}$ of the nitrate supply to the euphotic zone, with the lowest $\delta^{15}\text{N}$ occurring in the subtropical gyre (Marconi et al., 2017). Ocean circulation changes might alter the $\delta^{15}\text{N}$ of the nitrate supply to the Gulf of Maine by changing the relative importance of this low $\delta^{15}\text{N}$ subtropical nitrate relative to the nitrate imported to the surface at higher latitudes, and a $\delta^{15}\text{N}$ change in soft coral has been interpreted in this way (Sherwood et al., 2011). If this process were important in our cod $\delta^{15}\text{N}_{\text{oto}}$ decline, it would require a greater relative input of this subtropical nitrate to the Gulf of Maine under modern conditions. Existing hydrographic data do not show an obvious signature of this process (Townsend et al., 2010, 2015; Feng et al., 2016), but it cannot be ruled out.

Anthropogenic impacts on the $\delta^{15}\text{N}$ of fixed N supply to the Gulf of Maine euphotic zone must also be considered. Rivers in the present tend to deliver biologically available N with a high $\delta^{15}\text{N}$, leading to increases of up to 7‰ in macroalgae (McClelland et al., 1997; Savage, 2005) and increases of up to 4‰ for primary consumers and fish near rivers or wastewater point sources (Fry, 1999; Pruell et al., 2006; Corbett et al., 2015; Duprey et al., 2017). Explaining the decline in modern $\delta^{15}\text{N}_{\text{oto}}$ would require that this high $\delta^{15}\text{N}$ source is less important than in the 17th century, which seems unlikely. Moreover, several studies report that dilution with seawater reduces the $\delta^{15}\text{N}$ impacts from river- or wastewater-delivered waters within only 1–30 km from the point source (Savage, 2005; Pruell et al., 2006; Corbett et al., 2015; Duprey et al., 2017), arguing against a role in cod $\delta^{15}\text{N}_{\text{oto}}$ changes in general. Lastly, atmospheric N deposition, which has a low $\delta^{15}\text{N}$, typically constitutes a low contribution in nutrient-rich coastal and shelf systems. In summary, while baseline effects cannot be precluded with the existing data, a trophic effect from the decrease in fish size and/or a change in trophic level-at-size are currently our best explanations for the apparent Gulf of Maine cod $\delta^{15}\text{N}_{\text{oto}}$ decline since the 17th Century.

6.10. Future applications of $\delta^{15}\text{N}_{\text{oto}}$

Analyzing otoliths from 4000-year-old midden mounds or historical sites (Harris, 2011; Limburg et al., 2011), 9000-year old shelf sediments (Elder et al., 1996), or 33.7 Myr shelf sediments (Ivany et al., 2000) would allow for reconstructing pre-disturbance ecological conditions. Other than species interactions captured in the fossil record and

the physiology of ancient organisms indicating dietary preferences, there are few options for investigating the ecology of ancient oceans. Whether prehistoric otoliths still retain OM is as yet unknown. Many paleo-ecological studies use otoliths to reconstruct the species diversity or paleoecology of past oceans (Frizzell and Dante, 1965; Aguilera and Rodrigues de Aguilera, 2001; Schwarzhans et al., 2016), and some of these otoliths are exceptionally well preserved (Gierl et al., 2013). Possible studies with policy implications include examining changes in food webs resulting from climate change or from the arrival of European and American commercial fishing activities in the Western Atlantic. As described above, analyses of co-occurring fossils, especially of primary consumers such as bivalves or gastropods found in the same strata as otolith samples, might help to constrain baseline $\delta^{15}\text{N}$ for each time period.

$\delta^{15}\text{N}_{\text{oto}}$ has potential for investigating long term ecological patterns in populations on decadal and centennial time scales (e.g., Rowell et al., 2010; Sirot et al., 2017). Many countries with fisheries economies have otolith archives spanning the 20th century, due to long term government sampling programs for collecting biological data on fish populations, which often include the collection of otoliths for fish age determination. Pairing $\delta^{15}\text{N}_{\text{oto}}$ with stock indices, e.g., identifying dietary changes in spawning stocks leading up to recruitment of especially large year classes, is an intriguing possible application of this method.

Combining $\delta^{15}\text{N}_{\text{oto}}$ methods with other otolith chemical measurements would enhance the utility of each for investigating fisheries ecology. Otolith microchemistry is used widely to investigate migratory behavior, habitat residency, and population connectivity of wild fishes (Campana and Thorrold, 2001; Sturrock et al., 2012). $\delta^{15}\text{N}_{\text{oto}}$ can provide an ecological dimension due to the dependence of $\delta^{15}\text{N}_{\text{oto}}$ on diet and baseline. For example, migratory versus resident subpopulations (e.g., as reviewed by Secor, 2015) are likely to exhibit different $\delta^{15}\text{N}_{\text{oto}}$, whether due to dietary differences or baseline differences. Other examples are the use of $^{87/86}\text{Sr}$ to determine natal stream for Atlantic salmon (Kennedy et al., 1997, 2000; Barnett-Johnson et al., 2008) in freshwater systems, the use of element-to-calcium ratios, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in the otolith aragonite to determine nursery ground in the marine environment (e.g., Kerr et al., 2007; Wells et al., 2012; Rooker et al., 2016), and the use of otolith chemistry measurements to identify sub-population structure of fish populations otherwise known to be homogeneous based on genetics (Svedäng et al., 2010). Combining natal stream or nursery ground identity with $\delta^{15}\text{N}_{\text{oto}}$ would provide insights concerning whether fish trophic level, as a result of diet, influences why specific streams or nursery grounds are more or less productive (resulting in differential recruitment success). In general, dietary reconstruction using $\delta^{15}\text{N}_{\text{oto}}$ has great potential to provide ecological mechanisms for fish behavior when paired with the geographic or migratory information from otoliths.

Lastly, future optimization of micromilling with $\delta^{15}\text{N}_{\text{oto}}$ will be useful for ecological investigations in both the modern and past ocean. As many species undergo ontogenetic changes in diet or habitat, micromilling would provide many exciting applications for reconstructing fish behavior

and resource use. Analysis of early life history (otolith core) material may allow for tracking long term changes in nitrogen cycling at the base of the marine food chain, as juvenile fishes consume primary consumers such as copepods that integrate isotopic changes in baseline. The current minimum analytical requirement for this method is ~ 6 nmol, corresponding to ~ 0.4 mg of material for the most OM-poor species measured in the current study. This roughly equates to the ability to micromill otolith core and outer edge, with up to two time points in between depending on the species and the size of the otolith. While coarse resolution relative to $\delta^{18}\text{O}$ or laser ablation-based element:calcium measurements, the resulting ontogenetic information may be enlightening.

7. CONCLUSION

A two-step cleaning process (superficial cleaning followed by a secondary cleaning of crushed otolith powder) results in robust N content and isotope measurements for modern and fossil fish (here, focusing on Atlantic cod) with a long term analytical precision of 0.3‰. A minimum mass of 0.4 mg of otolith material for the lowest N content species investigated so far (Atlantic cod) is required for analysis, which may allow for multiple measurements from single otoliths in the future. Cleaning experiments resulted in a better understanding of the distribution of organic matter within otoliths as a repository of N isotopic information. Otoliths are not highly porous, and at least some of the organic matter in otoliths must be physically exposed (e.g., by crushing and powdering the otolith) before it is accessible to harsh oxidant solutions. At that point, a substantial quantity of OM is still preserved in the powder. For modern otoliths, indistinguishable $\delta^{15}\text{N}$ between cleaned intact otoliths and cleaned, powdered otolith material implies that OM that is lost as a result of crushing and cleaning does not differ isotopically from that retained after cleaning. Results from farmed fish and from a cohort of pink salmon with homogenous life history suggest that physiologically-induced variations in $\delta^{15}\text{N}_{\text{oto}}$ are minimal. Taken together, these results imply that otoliths are useful repositories for ecological investigations, including trophic or baseline reconstruction or differences in baseline experienced by different groups of the same species. Lastly, for at least some fossil otoliths, cleaning is required to avoid potential artifacts associated with alteration of OM and results in N content typical of modern otoliths, indicating the usefulness of $\delta^{15}\text{N}_{\text{oto}}$ in fossil samples.

Otolith chemistry has greatly advanced our understanding of fish habitat and behavior. Most of the established otolith chemistry methods do not provide information on fish diet. The advantages of the N isotopic analysis method introduced here derive from its high sensitivity, which allows for individual otoliths to be analyzed and for intensive cleaning of the otolith material to avoid artifacts from foreign organic matter or diagenetic alteration. When combined with existing otolith microchemistry methods for environmental reconstruction, these data have great potential to inform our understanding of marine and freshwater

environmental and food web changes on various time scales.

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AUTHOR CONTRIBUTIONS

JLD, BBW, DMS, XTW, OPJ designed experiments. JLD conducted experiments. JLD conducted statistical analyses. JLD, BBW, DMS, XTW, OPJ interpreted data. JLD, DMS, and BBW wrote the paper.

APPENDIX A. SUPPLEMENTARY MATERIAL

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.gca.2018.01.001>.

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