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

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ABSTRACT

The nitrogen isotopic composition ($\delta^{15}N$) of otolith-bound organic matter (OM) is a potential source of information on dietary history of bony fishes. In contrast to the $\delta^{15}N$ of white muscle tissue, the most commonly used tissue for ecological studies, the $\delta^{15}N$ of otolith-bound OM ($\delta^{15}N_{oto}$) provides a record of whole life history. More importantly, $\delta^{15}N_{oto}$ can be measured in contexts where tissue is not available, for example, in otolith archives and sedimentary deposits. The utility and robustness of otolith $\delta^{15}N$ 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 $\delta^{15}N_{oto}$ 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 $\delta^{15}N_{oto}$ for ecological studies. For three salmonid species, left and right otoliths from the same fish are indistinguishable. We find that the $\delta^{15}N_{oto}$ of pink salmon (*Oncorhynchus gorbuscha*) is related to the size of the fish for this species. We find that intra-cohort $\delta^{15}N_{oto}$ 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 $\delta^{15}N_{oto}$ will be valuable for population-level studies. Lastly, our protocol yields reproducible data for both $\delta^{15}N_{oto}$ and otolith N content in 17th century Atlantic cod otoliths. All told, the results of this study bode well for the utility of otolith-bound $\delta^{15}N$ for investigating the environment and ecology of modern and past fish.

KEY WORDS

Fish otolith, biogenic aragonite, nitrogen isotopes, Atlantic cod

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 for 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; McMahon et al., 2013). However, the white muscle tissue $\delta^{15}N$ ($\delta^{15}N_{wmt}$) 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).

104 Otoliths are used for determining age in fishes; over one million fish are aged
105 each year by fisheries biologists for the purposes of fisheries stock assessments and
106 biological studies (Campana and Thorrold, 2001). Additionally, the field of otolith
107 microchemistry uses the concentrations of trace elements (e.g., Mg, Mn, Fe, Sr, Ba) or
108 isotopes (e.g., $\delta^{18}O$, $\delta^{13}C$, $^{87}/^{86}Sr$, $\Delta^{14}C$) in the mineral fraction of otoliths over years of
109 the fish's life or among different fish groups or individuals to reconstruct fish movement
110 or origin, largely based on the chemical "fingerprints" of different estuaries, rivers, or
111 oceanic provinces (Kalish, 1989; Campana and Thorrold, 2001; Sturrock et al., 2012).
112 Importantly, most of these studies focus on the chemistry of the mineral matter ($CaCO_3$)
113 as opposed to that of the organic matter (OM) (with the important exceptions of
114 Vandermyde and Whitledge, 2008; Rowell et al., 2010; McMahon, Fogel, et al., 2011;
115 McMahon, Berumen, et al., 2011; McMahon et al., 2012; Grønkjær et al., 2013; Sirot et
116 al., 2017). The annual growth bands ("annuli") that allow for enumeration of fish age
117 result from varying ratios of organic-to-mineral content, with periods of faster somatic
118 growth corresponding to otolith regions with a lower concentration of OM. This OM is
119 the substrate for $\delta^{15}N$ measurements in otoliths.
120 Efforts to measure otolith $\delta^{15}N$ have been limited by the small concentrations of
121 OM in otoliths (usually < 1-10% by mass; Degens et al., 1969; Borelli et al., 2001).
122 Previous studies ranged from distinguishing between agricultural and pristine watershed
123 origin (Vandermyde and Whitledge, 2008), to investigating prehistoric trophic
124 relationships (Rowell et al., 2010), to identifying the diet of Atlantic cod (Grønkjær et al.,
125 2013). Most studies required large or multiple otoliths to obtain a single measurement,

126 due to their use of the well-tested but relatively low-sensitivity approach of combustion
127 of OM to N_2 (Vandermyde and Whitledge, 2008; Rowell et al., 2010; Grønkjær et al.,
128 2013; Sirot et al., 2017). Grønkjær et al. (2013) and Sirot et al. (2017) include a
129 dissolution centrifugation step for separation of the water-soluble and -insoluble fractions
130 of OM prior to combustion to N_2 and subsequent isotope analysis.

131 In addition to the challenge of the low concentration of OM in otoliths, the fish-
132 native origin and isotopic preservation of the OM must also be assured. In fossil otoliths,
133 diagenetic processes have the potential to cause loss of N-containing components of OM.
134 Loss of N-containing components is frequently associated with isotopic fractionation that
135 results in higher $\delta^{15}N$ of the remaining OM (Macko et al., 1986; Gannes et al., 1998;
136 Hannides et al., 2013), although $\delta^{15}N$ can also be lowered and/or simply made more
137 variable under some conditions (Altabet, 1988; Altabet et al., 1991; Lehmann et al., 2002;
138 Tremblay and Benner, 2006; Robinson et al., 2012). Additionally, exogenous N may be
139 added to sedimentary materials during their accumulation, and some of this exogenous N
140 may be mobile in the diagenetic setting. Thus, diagenesis can introduce uncertainty in the
141 interpretation of N isotope measurements in terms of past environmental, ecological, or
142 physiological processes. Studies of fossil carbonate-bound OM in other biogenic
143 structures such as coral, foraminifera, and ostracods have found a preliminary cleaning
144 step to be essential for the removal of altered endogenous OM and exogenous OM (e.g.,
145 Ingalls et al., 2003; Ren et al., 2009; Bright and Kaufman, 2011; Wang et al., 2014).
146 Previous work on the $\delta^{15}N$ of fossil otoliths has generally not taken steps to address or
147 avoid diagenetically altered or exogenous OM (Rowell et al., 2010), in part because

148 cleaning techniques further reduce the amount of otolith-native OM available for isotopic
149 analysis. One goal of the current study was to investigate the efficacy and necessity of a
150 pre-cleaning treatment to remove diagenetically altered and exogenous N, leaving only
151 otolith-native N for $\delta^{15}N$ analysis.

152 Lastly, we demonstrate the application of otolith N isotopic composition for
153 ecological and oceanographic studies. The $\delta^{15}N$ of metazoans records two factors: the
154 $\delta^{15}N$ of the primary producers at the base of the food web and the trophic level of the
155 organism.

156 The $\delta^{15}N$ of the primary producers at the base of the food web, often referred to as
157 baseline $\delta^{15}N$, is controlled by large scale factors such as the $\delta^{15}N$ of nitrate supply to the
158 euphotic zone (Ren et al., 2009; Ren et al., 2012) and the degree of nitrate consumption
159 by phytoplankton (Wada and Hattori, 1976; Altabet et al., 1991; Altabet and Francois,
160 1994), as well as by more specific factors such as the phytoplankton forms that ultimately
161 support the heterotrophic species being studied (Fawcett et al., 2011; Fawcett et al.,
162 2014). Differences in primary producer $\delta^{15}N$ are imprinted on primary consumers, for
163 example copepods, and subsequently propagate up the food chain (Hobson, 1999;
164 McMahon et al., 2013; Dunton et al., 2017). Differences in baseline $\delta^{15}N$ and $\delta^{13}C$ are
165 used to track marine migrations of species if animals reside in isotopically distinct
166 environments for long enough for their tissues to record that signal (Schell et al., 1998;
167 Schell, 2001; Newsome et al., 2010; McMahon et al., 2013)..

168 The trophic factor can be summarized by the aphorism, “you are what you eat,
169 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 over time 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 $\delta^{15}N$ 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 $\delta^{15}N$ is the result of diet, baseline, or a combination of the two factors, baseline can be constrained by measuring primary producer or primary consumer $\delta^{15}N$ 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 $\delta^{15}N$ (McClelland and Montoya, 2002; Chikaraishi et al., 2009).

To measure $\delta^{15}N_{oto}$, 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 $\delta^{15}N_{oto}$ as a proxy for fish diet: (1) Is the proxy adequately

192 precise to record differences within and across individual fish and among fish groups? (2)
193 Is $\delta^{15}N_{oto}$ consistent with existing trophic level information? (3) Is $\delta^{15}N_{oto}$ robust against
194 diagenetic alteration for historical otolith samples? $\delta^{15}N_{oto}$ of historical fishes may
195 provide insight into past food web structure and environmental conditions. This potential
196 application is preliminarily investigated in Atlantic cod (*Gadus morhua*) by comparison
197 of modern and fossil otoliths. To gain perspective on human impacts on modern fisheries,
198 $\delta^{15}N_{oto}$ can provide information about fish and food webs under pre-disturbance
199 conditions.
200
201 **2. METHODS**
202 The $\delta^{15}N_{oto}$ method reported here consists of eight steps (Fig. A1): initial cleaning
203 of the whole otolith, crushing of the whole otolith, oxidative cleaning of "exposed" OM,
204 acid dissolution of the cleaned otolith aragonite, oxidation of the released ("carbonate-
205 bound") OM to nitrate, quantitative conversion of the nitrate to N_2O, automated
206 extraction, cryogenic and gas chromatography-based purification, and isotopic analysis of
207 the N_2O. The latter steps, which yield isotopic measurements of nitrate, are often
208 summarized as the "bacterial conversion" or "denitrifier method" (Sigman et al., 2001;
209 Casciotti et al., 2002; Mcilvin and Casciotti, 2011; Weigand et al., 2016), which is 200-
210 fold more sensitive than standard approaches involving combustion to N_2 and initiated
211 the N_2O-based approach for isotopic analysis of diverse N forms.
212
213 ***2.1 Preparation and initial cleaning of whole otoliths***

214 Fish (mostly Atlantic cod and salmonids) were obtained from various sources
215 including local fish markets and fish hatcheries (Table 1). Only sagittal otoliths were
216 used. Otoliths used for all experiments below were soaked for 24 h in 10 mL sodium
217 hypochlorite (NaOCl, 10-15% available chlorine) and rinsed three times in deionized
218 water (DIW). They were then transferred to pre-combusted 12 mL borosilicate glass vials
219 and dried for up to 24 h at 30ºC or until completely dry, visually inspected using a
220 microscope to ensure complete removal of tissues, and weighed to ± 0.01 mg.
221
222 ***2.2 Procedures for oxidative cleaning of otolith grains***
223 Cleaned otoliths were crushed and homogenized using a mortar and pestle. The
224 mortar and pestle were cleaned with dust-free spray air and dilute hydrochloric acid
225 (10%) between samples. Grains were ground to 38-150 µm (determined by sieving)
226 unless otherwise stated. Resulting otolith powder was soaked for 24 h in sodium
227 hypochlorite in 15 mL polypropylene centrifuge tubes. Tubes for sodium hypochlorite
228 cleaning were oriented horizontally on a shaker (IBI Scientific) for 24 h. Samples were
229 then rinsed three times with DIW, using centrifugation (3 min at 2900 rcf) to prevent loss
230 of crushed material while removing supernatant between rinses. From this stage forward,
231 the N blank associated with the DIW used for cleaning or included in the persulfate
232 oxidation solution was minimized by a final low-temperature distillation (Savillex
233 Corporation, Minnetonka, MN). This distillation represents an additional step to
234 minimize background concentrations of organic matter in the DIW used for reagent
235 solutions that are subsequently added to samples. Minimizing the relative contribution of

236 nitrogen from non-sample sources allows for analysis of relatively small N
237 concentrations in otoliths.
238 After cleaning and rinsing, otolith powder was transferred to pre-combusted 12
239 mL glass vials, and excess water was removed using pre-combusted glass Pasteur
240 pipettes fitted to a diaphragm vacuum pump. Samples were dried for 12-48 h at 30ºC in a
241 drying oven reserved for low-N samples. Once completely dry, otolith powder was
242 weighed into 4 mL pre-combusted borosilicate glass vials with freshly cleaned and dried
243 Teflon-lined caps. Final masses were 3-4 mg (± 0.01 mg; MettlerToledo) unless
244 otherwise specified.
245
246 ***2.3 Isotopic analysis***
247 Cleaned and dried otolith powder was dissolved, oxidized, and analyzed as in
248 Wang et al. (2014), as summarized here. A 50 µL solution of 4 N Optima grade
249 hydrochloric acid was used to dissolve the $CaCO_3$; sample vials were shaken and visually
250 inspected to ensure complete dissolution of $CaCO_3$. One mL of freshly combined
251 persulfate oxidizing reagent (POR) (1 g of 4X recrystallized potassium persulfate and 1 g
252 ACS grade sodium hydroxide into 100 mL DIW) was added to the sample vials and
253 autoclaved for 90 min at 120ºC to convert organic nitrogen to nitrate (Solorzano and
254 Sharp, 1980; Bronk et al., 2000). The reagent mixture is unstable and thus was used
255 immediately after combination. After cooling, precipitate was removed by centrifugation
256 for 4 min at 4600 rcf. The supernatant was transferred to new precombusted 4 mL
257 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 $\delta^{15}N$, the precision of the bacterial conversion and isotopic analysis is < 0.05‰ for nitrate reference solutions (the precision associated with $\delta^{15}N_{oto}$ 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 $\delta^{15}N$ 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 $\delta^{15}N_{blank}$ for each POR batch. Blank corrections were conducted as per (Gelwicks and Hayes, 1990):

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

where M refers to mass, and the term $M_{sample} = M_{mix} - M_{blank,}$ and where M_{mix}, $\delta^{15}N_{mix}$, and M_{blank} were measured directly via GC-IRMS. $\delta^{15}N_{blank}$ was measured directly and was also 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 $\delta^{15}N$ 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). A cod otolith standard made of homogenized sagittal otoliths of four individual Atlantic cod caught on 13 November 2014 and landed in Chatham, MA was made 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 only. Otolith powder (3.15-3.75 ± 0.01 mg) was added to 15 mL polypropylene centrifuge tubes and cleaned using sodium hypochlorite. Samples were either maintained at room temperature (22ºC) or heated to 60ºC using a water bath. Centrifuge tubes for both room temperature and heated experiments were shaken vigorously every 6 hours. 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 60ºC. 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 $\delta^{15}N_{oto}$ 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 120ºC. 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,

346 with a centrifuge step between rinses to concentrate otolith powder and prevent loss of
347 sample, and transferred to precombusted 12 mL glass vials. Extra water was removed
348 using pre-combusted glass pipettes. Samples were then dried in a drying oven at 30ºC for
349 12 h or until completely dry (usually 12-48 h). Analysis of $\delta^{15}N_{oto}$ was conducted as
350 described above (*Section 2.3*).
351
352 ***3.4 Effect of cleaning on fossil otoliths***
353 The necessity of removing exposed OM for midden-deposited otoliths was tested.
354 Two broken otoliths (fractured *in situ,* prior to archaeological excavation; Fig 1) were
355 studied to address whether there was a measurable difference with and without cleaning
356 of their ground powder (Fig. 2). The fossil otoliths were excavated from Smuttynose
357 Island, the site of a commercial fishing station that shipped dried cod to European
358 markets during the 17th century (Appendix A for full site description). Fish heads were
359 routinely discarded into trash sites (middens) along with other contemporaneous artifacts
360 including other fish biological remains, ceramics, and pipe stems (Robinson, 2012;
361 Moyer et al., 2015). Because the historical otoliths had been buried for over 300 years in
362 sediments, and were in various states of preservation (Fig. 1), these samples were treated
363 with two additional initial cleaning steps. For clay removal, otoliths were soaked in 2 ‰
364 sodium polyphosphate and sonicated for 5 minutes in an ultrasonication bath. Next,
365 otoliths were soaked in bicarbonate-buffered dithionite citrate (pH ~7.5, 1 h at 80ºC water
366 bath) as a reductive cleaning agent to remove metals (Mehra and Jackson, 1958). This
367 was followed by an oxidative cleaning with sodium hypochlorite for removal of external

368 OM (Wang et al., 2014), as per modern otoliths (*Section 2.1*). This external cleaning
369 resulted in fossil otoliths that were devoid of discoloration and sediment (Fig. A2). After
370 external cleaning, the otoliths were snapped to yield half of an intact otolith (Hu and
371 Todd, 1981) for subsequent comparison of otolith halves, as opposed to comparison of
372 differently-broken otoliths. In breaking the otolith, ages were also discernable: Otolith A
373 was larger, from a fish ≥ 9 years old (outer-most growth bands were obscured and
374 indistinguishable), and Otolith B was smaller, from a 3-year-old fish (all growth bands
375 were discernible). The sizes of otolith A and B are similar to those of otoliths in Fig. 1c
376 and d. Otolith halves from each fish were crushed and homogenized with a mortar and
377 pestle and split into two groups per otolith: "Surface and grain cleaning" and "Surface
378 cleaning only" (n = 3 - 4 replicate subsamples per treatment for each otolith) (Fig. 2).
379 Surface and grain cleaning otolith powder subsamples were weighed to 4.5 - 5 mg (±
380 0.01 mg) and treated as above (*Sections 2.2 - 2.4*) with sodium hypochlorite cleaning of
381 otolith grains for 12 hours followed by DIW rinses, dissolution, oxidation, and $\delta^{15}N$
382 analysis. Surface cleaning only powder was not treated with sodium hypochlorite at all
383 after crushing and instead subsamples were weighed to 4.5 - 5 mg (± 0.01 mg), directly
384 dissolved and oxidized, and then analyzed by the usual protocol as above (*Sections 2.3 -*
385 *2.4*).
386
387 **4. EXAMPLES OF ECOLOGICAL APPLICATIONS**
388 ***4.1 Isotopic identity of wild and farm-raised salmonids***
389 A pilot study was conducted to determine the variability of $\delta^{15}N_{oto}$ in farm-raised

390 and wild-caught fish populations, between left and right otoliths from the same
391 individuals, and between intact versus crushed otoliths (Table 2). Otoliths were dissected
392 from farm-raised brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*)
393 provided by Musky Trout Hatchery, Asbury, NJ on 24 July 2014. For comparison,
394 otoliths of wild pink salmon (*Oncorhynchus gorbuscha*) were provided by ADF&G,
395 Cordova, Alaska, from fish harvested in the commercial fishery. These fish were caught
396 on 22 August 2008 from two ADF&G management districts (Statistical Areas 222 and
397 226; see Fig. A3 for map of statistical areas) separated by approximately 40 miles in
398 Prince William Sound, Alaska.
399 Otoliths of thirteen brown trout and ten rainbow trout were analyzed. Five pairs of
400 pink salmon otoliths from each of two ADF&G districts (a total of ten individuals) were
401 analyzed. For all three species, left (L) and right (R) otoliths from the same fish were
402 compared to test whether $\delta^{15}N_{oto}$ varies between sagittal otoliths from the same fish.
403 Additionally, one otolith from each of three pink salmon pairs was crushed to compare
404 the $\delta^{15}N_{oto}$ of crushed otolith material with that of an intact (uncrushed) otolith that
405 otherwise underwent the same cleaning. Lastly, for both farmed- and wild-caught fish,
406 the relationship between fish size and $\delta^{15}N_{oto}$ was investigated.
407
408 ***4.2 Nitrogen isotopic composition of Atlantic cod white muscle tissue***
409 For comparison with $\delta^{15}N_{oto}$, approximately 1 cm^3 of white muscle tissue per fish
410 was freeze dried for 24 hours, then crushed and homogenized with a mortar and pestle,
411 and finally weighed to 1 ± 0.2 mg into tin capsules. The $\delta^{15}N$ of white muscle tissue

412 ($\delta^{15}N_{wmt}$) was determined for each sample using an Isoprime 100™ isotope ratio mass
413 spectrometer interfaced in continuous flow with an elemental analyzer (Vario ISOTOPE
414 cube™, Elementar) at Princeton University. The average standard deviation of replicate
415 subsamples was 0.04‰.
416
417 ***4.3 Comparison of historical and modern Atlantic cod***
418 $\delta^{15}N_{oto}$ was compared among Atlantic cod from four sources (see also Table 1): 1)
419 fossil otoliths of commercially caught cod from midden deposits of Smuttynose Island,
420 Maine (described above), dated to the 17th century no earlier than 1630 using Lewis
421 Binford Analysis (Binford, 1962; Robinson, 2012); 2) modern cod, landed in Chatham,
422 MA, from a Gulf of Maine sector fishing boat, on November 13th, 2014, and obtained
423 from a fish market (Nassau Seafood, Princeton, NJ); 3) otoliths of two-year-old modern
424 cod collected by the NOAA Fisheries Northeast Fisheries Science Center (NEFSC) from
425 Georges Bank, USA, during fisheries-independent research surveys of the NEFSC
426 Fishery Biology Program in 1981, 1984, 1987, and 2013 between September and
427 November of each year, and 4) lastly, five-year-old farmed cod that had lived their entire
428 post-larval lives in a controlled aquaculture setting at the University of Maine Center for
429 Cooperative Aquaculture Research (CCAR), where the cod were fed commercially
430 formulated aquafeed (Skretting Europa). Only unbroken otoliths were used. Historical
431 otoliths were treated with two additional initial cleaning steps as described above (see
432 *Section 3.4* above).
433 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 41.5ºN - 44.8ºN 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 $\delta^{15}N_{oto}$ 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 ± 1σ 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 (60ºC) sodium hypochlorite (Fig. 4). $\delta^{15}N_{oto}$ did not significantly differ between short (0.5-1 h at room temperature) and longer (6-48 h at room temperature) cleaning duration, although the variance (standard deviation) improved after N content stabilized at 6 hours (7.1 ± 0.6‰ at 0.5-1h to 7.2 ± 0.3‰ at ≥ 6 h; $p = 0.74$). N

content of uncleaned (0.5 h) otolith powder was 62% higher than cleaned otolith powder after 6 hours (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 60ºC, $\delta^{15}N_{oto}$ was not statistically distinct, including samples cleaned for 6 hours or more, the time after which N content stabilized for both heated and room temperature treatments (6.9 ± 0.2‰ heated, 7.2 ± 0.3‰ 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.

3) Grain size: Grain size had no significant effect on $\delta^{15}N_{oto}$ ($p > 0.40$ for all grain sizes) for either POR- or sodium hypochlorite-cleaned CDS (Fig. 5). However, $\delta^{15}N_{oto}$ 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). When 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 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; $p = 0.05$). Thus, the preponderance of evidence indicates that there is no dependence of measured N content on ground otolith grain size.

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

478 in $\delta^{15}N_{oto}$ between POR- and sodium hypochlorite-cleaned CDS (Table 3). However, for
479 PSS and QSN, POR cleaning resulted in lower $\delta^{15}N_{oto}$ by 0.8 ‰ and 0.4 ‰ respectively.
480 N content was 10% higher for POR-treated CDS compared to sodium hypochlorite-
481 treated samples. For PSS and QSN, N content of POR-treated samples was higher by
482 47% and by 342% respectively compared to sodium hypochlorite-treated samples (Table
483 3). These starkly higher N contents for cleaning by POR and sodium hypochlorite very
484 likely derive from aragonite dissolution and calcite reprecipitation at the high temperature
485 of POR cleaning (*Section 6.2*).
486
487 ***5.2 Historical otolith testing***
488 For Otolith A, both $\delta^{15}N_{oto}$ and N content decreased significantly after cleaning (p
489 < 0.05 for both $\delta^{15}N_{oto}$ and N content), but no significant change in $\delta^{15}N_{oto}$ or N content
490 was observed for Otolith B ($p = 0.11$ for $\delta^{15}N_{oto}$, $p = 0.35$ for N content). With cleaning,
491 $\delta^{15}N_{oto}$ changed from 10.3 ± 0.2 ‰ to 9.5 ± 0.3 ‰ for fossil Otolith A and from 7.9 ± 0.2
492 ‰ to 7.5 ± 0.4 ‰ for fossil Otolith B (Fig. 6). The N content decreased from 19.8 ± 1.6
493 to 15.8 ± 0.7 nmol N mg^{-1} after cleaning for Otolith A (uncleaned powder had 25%
494 higher N content than cleaned powder for Otolith A), but showed no difference for
495 Otolith B, in which the uncleaned powder was 14.3 ± 0.3 and the cleaned powder was
496 14.6 ± 0.5 nmol N mg^{-1}. The crushing without subsequent cleaning performed in this test
497 could introduce contaminant N. However, Otolith B has indistinguishable N content
498 between uncleaned and cleaned treatments, and the four uncleaned subsamples from
499 Otolith A are uniformly higher in N content relative to cleaned subsamples. Therefore,

500 the differences in N content and $\delta^{15}N_{oto}$ between Otolith A and Otolith B are best
501 interpreted in terms of otolith-associated organic matter rather than contamination during
502 grinding.
503
504 ***5.3 Otolith-bound organic matter in wild and farm-raised fish***
505 For the farm-reared brown trout and rainbow trout, which were fed the same food,
506 average $\delta^{15}N_{oto}$ was 11.5 ± 0.3‰ (n = 13) and 11.3 ± 0.4‰ (n = 10), respectively (Fig. 7),
507 and $\delta^{15}N_{oto}$ of the two species were not significantly different (p = 0.28). Average N
508 content in brown trout was greater than in rainbow trout (21.8 ± 2.4 nmol N mg^{-1} vs. 18.1
509 ± 3.9 nmol N mg^{-1}, $p < 0.05$; Suppl. Table A1).
510 Average $\delta^{15}N_{oto}$ for wild pink salmon was 14.5 ± 0.4 ‰ (Fig. 7). The average for
511 crushed otoliths was 14.4 ± 0.6 ‰ (n = 3) and for intact otoliths was 14.5 ± 0.4 ‰ (n =
512 16), with no difference in $\delta^{15}N$. The average $\delta^{15}N_{oto}$ difference between crushed and
513 intact otoliths from the same fish was -0.2 ± 0.3‰ (crushed-intact; Fig. 8; n =3).
514 Statistical Area 222 was not significantly different than Area 226 (p = 0.85). For N
515 content, crushed pink salmon otoliths yielded an average N content of 17.1 ± 2.0 nmol N
516 mg^{-1} whereas intact otoliths contained 32.0 ± 3.2 nmol N mg^{-1}, or 87% more N than
517 crushed otoliths after cleaning.
518 Otolith weight for whole pink salmon otoliths ranged from 2.5 to 4.2 mg,
519 averaging 3.33 mg (Fig. 9). Despite this small mass analyzed, the average standard
520 deviation of L and R otoliths from the same fish was only 0.2 ‰ (Fig. 8). In general,
521 when combining across all three salmonid species (Fig. 8), L vs. R otoliths were not

522 significantly different (p = 0.998) and were highly correlated (Pearson correlation, r =
523 0.99, p < 0.001). Focusing on the pink salmon alone, L vs. R otoliths were not
524 statistically different (Fig. A4; p = 0.82) and were again highly correlated (Pearson
525 correlation, r = 0.83, p < 0.001). There was not a significant correlation between otolith
526 size and otolith N content (Pearson correlation, r = -0.35, p = 0.35). However, the
527 relationship between otolith size and $\delta^{15}N_{oto}$ for wild pink salmon was significant (Fig 6;
528 Pearson correlation, r = 0.88, p < 0.001) even within a very small size range, as would be
529 expected given that otolith size is correlated with fish size and that fish size is correlated
530 with trophic level for this species (Aydin et al., 2005). In contrast, there was no
531 relationship between otolith size and $\delta^{15}N_{oto}$ for farm-reared fish (Fig. A5; Pearson
532 correlation, r = 0.04, p = 0.87), consistent with the lack of potential for trophic level
533 change or baseline change with increasing size in the fish farm setting.
534
535 ***5.4 Comparison of $\delta^{15}N_{wmt}$ to $\delta^{15}N_{oto}$***
536 $\delta^{15}N_{wmt}$ averaged 15.1 ± 0.7 ‰ for modern cod and 12.8 ± 0.5 ‰ for farmed cod.
537 $\delta^{15}N_{wmt}$ and $\delta^{15}N_{oto}$ from the same fish were linearly correlated (Fig. 10; Pearson
538 correlation, r = 0.80, p < 0.001) with a slope of 0.69 (± 0.33 95% confidence interval) and
539 intercept of -2.7 (± 4.78 95% confidence interval). $\delta^{15}N_{wmt}$ was on average 7.3 ± 0.7 ‰
540 higher than $\delta^{15}N_{oto}$ from the same fish individual, and there was no significant difference
541 in the $\delta^{15}N_{wmt}$ to $\delta^{15}N_{oto}$ offset for wild versus farmed cod (7.5 ± 0.7 ‰ offset compared
542 to 6.7 ± 0.5‰ offset; p = 0.07). Based on the above regression, average $\delta^{15}N_{wmt}$ was
543 predicted to be 18.6 ‰ from fossil otoliths.

544

545 ***5.5 Comparison of 17th and 21st century Atlantic cod***

546 Historical Atlantic cod $\delta^{15}N_{oto}$ averaged 10.0 ± 0.6 ‰ (ranging from 9.6 to 10.7
547 ‰), while modern commercially harvested cod $\delta^{15}N_{oto}$ averaged 7.9 ± 0.9 ‰ (ranging
548 from 6.7 to 9.2 ‰) and the two groups were significantly different (Fig. 11, Fig. A6; $p <$
549 0.001). Wild age-2 Georges Bank (GB) cod from NEFSC autumn research survey were
550 8.1 ± 0.7 ‰ (ranging from 6.9 to 9.8 ‰) which was indistinguishable from commercially
551 harvested Gulf of Maine (GOM) cod ($p = 0.52$) but significantly different from fossil cod
552 $\delta^{15}N_{oto}$ ($p < 0.001$). For farm-raised cod, the average $\delta^{15}N_{oto}$ was 6.2 ± 0.3 ‰ (ranging
553 from 5.8 to 6.4 ‰). Using the $\delta^{15}N_{wmt}$ to $\delta^{15}N_{oto}$ linear regression above (*Section 5.4*),
554 predicted $\delta^{15}N_{wmt}$ of historical cod may have ranged from 18.0 - 19.5 ‰. The N content
555 was 14.6 ± 1.1 nmol N mg^{-1} and 16.1 ± 1.6 nmol N mg^{-1} for historical and modern GOM
556 cod, respectively, and they were not significantly different ($p = 0.11$). GB cod were 16.3
557 ± 1.6 nmols N mg^{-1} and were not significantly different from modern GOM cod ($p =$
558 0.84) or fossil GOM cod ($p = 0.06$). For farmed cod, N content was 21.3 ± 1.7 nmol N
559 mg^{-1}. Since the farm-raised cod had a food source unrelated to that available in the Gulf
560 of Maine, only the N content results are relevant for comparison to the wild cod. The
561 average otolith mass for historical, wild caught, and farmed cod was 791.68 ± 254.03 mg,
562 533.55 ± 86.71 mg, and 480.81 ± 21.19 mg. If converted to fish size, based on a
563 relationship of otolith mass to fish length from modern fish (Fig. A7; n = 463), we find
564 that this translates to a range of 64 cm to 97 cm (mean 84 ± 15 cm) for historical cod, and
565 a range of 58 cm to 78 cm (mean 70 ± 7 cm) for wild modern cod.

566

567 **6. DISCUSSION**

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

572

573 **6.1. Effects of oxidative cleaning on otolith powders from modern and fossil otoliths**

574 **6.1.1 Modern otoliths**

575 The organic fraction of otoliths, often called the "organic matrix", is composed of
576 insoluble, collagen-like proteins and soluble, high molecular weight organic molecules
577 that together are thought to control the structure and morphology of the mineral fraction
578 (Degens et al., 1969; Söllner et al., 2003; Falini et al., 2005), as in other biogenic
579 carbonates (Weiner, 1984; Belcher et al., 1996). The configuration of the organic matrix
580 and its interaction with the mineral fraction in otoliths and other biominerals is an active
581 field of study (DeVol et al., 2015; Wojtas et al., 2015; Mao et al., 2016). Organic matter
582 (OM) concentration varies at multiple spatial scales within the otolith, from nanometers
583 to millimeters (Dunkelberger and Dean, 1980; Watabe et al., 1982; Morales-Nin, 1986;
584 Dauphin and Dufour, 2008). The molecular-level associations are likely diverse, probably
585 covering a spectrum between truly intracrystalline and intercrystalline, and are beyond
586 the scope of this study. Here, "protected" OM refers to OM that is sufficiently trapped
587 within the crushed otolith grains that it can only be accessed once the mineral fraction is

588 dissolved. In contrast, “exposed” OM is OM that is removed by treatment with a harsh
589 oxidant dissolved in water. Accordingly, the distinction between protected and exposed
590 OM will depend, for example, on whether the otolith was ground prior to cleaning.
591 In the present study, we evaluated the scale at which otolith OM is accessible to a
592 harsh oxidant by analyzing crushed otolith powders of grain sizes between 60 µm and
593 425 µm. We found that otolith N content and $\delta^{15}N$ were unchanged regardless of surface-
594 area-to-volume exposed to the sodium hypochlorite prior to crushing and that the
595 standard deviation of replicate subsamples was not affected by grain size, except for the
596 largest grain sizes, which had higher standard deviations likely due to the lower number
597 of grains per sample. The fact that the cleaning methods are efficient across all grain sizes
598 implies that the OM composition is broadly consistent across the otolith. At the same
599 time, smaller grain sizes increased the precision, which suggests heterogeneity at some
600 scales within the otolith; as otoliths record the entire life history of fishes, some
601 heterogeneity over fish lifetime is expected.
602 One concern is that extended oxidative cleaning might remove a significant
603 fraction of the otolith-native OM. We found that the operationally defined protected OM
604 remains so across a time course of exposure to sodium hypochlorite (Fig. 4). N content
605 stabilized after only six hours, without evidence of further decline out to 36 hours. This
606 suggests that otolith grains are not porous on a scale that allows the cleaning methods to
607 continuously access otolith-native OM. Additionally, sodium hypochlorite cleaning at
608 60°C yielded a final N content that was indistinguishable from sodium hypochlorite
609 cleaning at room temperature, although the heating treatment resulted in faster removal of

610 exposed N, stabilizing after 1 hour rather than 6 hours. This is consistent with heated
611 versus room temperature cleaning of ostracod valves (Bright and Kaufman, 2011) and
612 ratite eggshell (Brooks et al., 1990; Crisp et al., 2013), suggesting the existence of a pool
613 of well-protected OM in at least some biogenic minerals.
614 Uncleaned cod otolith grains (CDS) have up to 62% more N compared to cleaned
615 otolith grains (Fig. 4). Intact pink salmon otoliths contained 87% more N than crushed
616 otoliths. Cleaning removes this additional N, leaving only the operationally defined
617 protected OM for subsequent analysis. The results of the time tests and of the intact vs.
618 crushed experiment for pink salmon show that exposed and protected OM provide
619 indistinguishable $\delta^{15}N$ (Figs 2 and 3). This argues that, in non-fossil otoliths, the two OM
620 classifications are not fundamentally different from one another other than in the degree
621 of protection afforded by the mineral. More broadly, the overall protected nature of the
622 otolith-bound OM is consistent with studies on coral (Ingalls et al., 2003; Wang et al.,
623 2014; Wang et al., 2015), foraminifera (Ren et al., 2009; Straub et al., 2013), and clam
624 shell (Crenshaw, 1972), wherein OM remains protected during continued exposure to
625 harsh oxidative cleaning.
626
627 **6.1.2 Fossil otoliths**
628 The efficacy of oxidative cleaning must also be assessed for fossil otoliths from
629 diagenetically active environments (Fig. 6). Diagenetic processes can alter the $\delta^{15}N$ of
630 OM, impeding interpretation in terms of primary biological or environmental processes.
631 Thus, diagenetically exposed OM, which may have been altered or contaminated by

632 diagenesis, should be removed prior to isotope analysis. Diagenetic N loss most often
633 elevates $\delta^{15}N$ (Robinson et al., 2012, and references therein). Under typical open ocean
634 conditions of low organic matter preservation and oxic bottom waters, OM buried in
635 marine sediments is higher than the $\delta^{15}N$ of the OM delivered to the seabed (Altabet and
636 Francois, 1994; Altabet, 2006). Similarly, the $\delta^{15}N$ of suspended OM in deep ocean
637 waters is ≥3‰ higher than the OM sinking into the ocean interior (Saino and Hattori,
638 1980; Altabet et al., 1991; Casciotti et al., 2008; Hannides et al., 2013). Evidence for
639 preferential diagenetic loss of OM with low $\delta^{15}N$ also comes from studies of relict
640 organic-rich layers in deep sea sediments (Möbius et al., 2010) as well as studies of soils
641 (Natelhoffer and Fry, 1988), peat bogs (Macko et al., 1990), and salt marshes (Fogel et
642 al., 1989).
643 The degree of isotopic alteration by diagenesis appears to depend on conditions.
644 Under the high OM preservation and low-oxygen conditions of certain isolated marine
645 basins and productive margin settings, smaller differences are observed between sinking
646 and buried OM $\delta^{15}N$ (Altabet et al., 1999; Ganeshram et al., 2000; Thunell et al., 2004;
647 Robinson et al., 2012). Studies of buried Mediterranean sapropels (Möbius et al., 2010)
648 and buried *Spartina* marsh grasses (Tremblay and Benner, 2006) also suggest that high
649 preservation and/or anoxic conditions can prevent a clear rise in $\delta^{15}N$ with diagenesis. In
650 contrast, substantial elevation of (≥ 5 ‰) is frequently observed in open ocean settings
651 (Altabet and Francois, 1994). In parallel, laboratory studies suggest variation in the
652 isotopic impact of diagenesis with redox condition (Lehmann et al., 2002). Moreover,
653 externally sourced N can also be added to sedimentary materials during deposition,

654 burial, and diagenesis, further overprinting the primary isotopic signal (e.g., Schubert and
655 Calvert, 2001; Meckler et al., 2008; Ren et al., 2009; Meckler et al., 2011). Given these
656 complexities, the only robust way to address it is to remove OM that may have been
657 exposed to diagenesis.

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

672 N content did not vary significantly as a function of cleaning for Otolith B.
673 Otolith B (similar to Fig. 1d) was missing its rostrum (end) potentially exposing a
674 pathway for both diagenetic fluids and our cleaning solutions to access the interior of the
675 otolith. One possible explanation is that the external cleaning (*Section 3.4*) was able to

676 access the exposed OM through the extant cracks in this broken otolith, resulting in the
677 leaching of all diagenetically altered or contaminated OM prior to the powder oxidative
678 cleaning step. An alternative explanation is that diagenesis had already removed all
679 affected N through the cracked fraction of the otolith (Fig. A8 shows details of a pitted
680 otolith).

681 The apparent difference in preservation quality of the two fossil otoliths
682 underlines the importance of the cleaning step. Whether lower N content was caused by
683 the external, surficial cleaning of the whole otolith or due to loss processes occurring in
684 sediments *in situ*, differences in preservation status here would have led to differences in
685 $\delta^{15}N$ if left unaddressed by the sodium polyphosphate, dithionite citrate, and sodium
686 hypochlorite cleaning. The standard protocol is to clean the intact otolith ("surficial
687 cleaning"), followed by crushing the otolith (in order to weigh the otolith if necessary,
688 and to homogenize and subsample the otolith) and cleaning the resulting otolith powder
689 with sodium hypochlorite ("otolith powder cleaning") (Fig. 2). For both damaged and
690 fully intact fossil otoliths, the standard $\delta^{15}N_{oto}$ protocol for otoliths (with two cleaning
691 steps, one before and one after otolith grinding) appears to effectively remove exposed
692 OM and results in N content that falls within the range expected for modern cod.
693

694 ***6.2 Differing effects of cleaning reagents on otolith stability***

695 The optimal cleaning is one that is harsh enough to remove diagenetically
696 exposed OM but not so harsh as to alter the $\delta^{15}N$ of the protected OM (Gaffey and
697 Bronnimann, 1993; Penkman et al., 2008; Bright and Kaufman, 2011). Given the long

698 history of persulfate-based oxidation as a strategy for completely oxidizing
699 environmental organic matter and its use at autoclave temperatures (Bronk et al., 2000),
700 we initially assumed that POR treatment would represent a harsher cleaning than that
701 using sodium hypochlorite at room temperature or 60°C. Based on this logic, if the two
702 cleanings were to yield different N content, we expected lower N content of POR-cleaned
703 powders relative to the sodium hypochlorite cleaning. However, we observed the
704 opposite tendency, with higher and more variable otolith-bound N content using POR,
705 and the difference was dramatic for two of three otolith standards: PSS and QSN (Table
706 3). We suspect that the N content difference is due to the recrystallization (or
707 dissolution/reprecipitation) of aragonite to calcite at the high temperature (up to 121ºC)
708 of the POR cleaning. Under these conditions, recrystallization of aragonite to calcite has
709 been observed and described in detail (Lécuyer, 1996; Pokroy et al., 2006; Ruiz-Agudo et
710 al., 2014; Staudigel and Swart, 2016). This transition may have resulted in the trapping of
711 otherwise-external OM on or near the surface of the otolith grains, for example, by the
712 development of a new coating of calcite, before that OM could be fully oxidized by the
713 reagent. The minimal effect on CDS N content may indicate that otolith aragonite formed
714 by cod is more stable than aragonite formed by pink salmon and queen snapper.
715
716 ***6.3 Variation in $\delta^{15}N_{oto}$ within individual fish***
717 $\delta^{15}N_{oto}$ in left and right otoliths of an individual fish differs less than $\delta^{15}N_{oto}$
718 among individuals, even within a small range of $\delta^{15}N_{oto}$ (~1‰; Fig. 8). Thus, an
719 individual fish has an isotopic identity that is recorded by both otoliths. This suggests that

720 the individual-to-individual $\delta^{15}N$ differences of less than 1‰ can be reconstructed with
721 $\delta^{15}N_{oto}$ (Fig. 8) and for very small otoliths (2.5 to 4.2 mg; Fig. 9). More mechanistically,
722 the L versus R comparison demonstrates that at least some forms of physiologically
723 driven variation in $\delta^{15}N_{oto}$ are too small to overprint the environment- and ecology-driven
724 variation in the $\delta^{15}N$ of individual fish in a population. Thus, even within a fairly
725 homogenous population, individual fish retain an isotopic identity, and individual-to-
726 individual $\delta^{15}N_{oto}$ differences of less than 1‰ can be reconstructed with $\delta^{15}N_{oto}$.
727
728 ***6.4 Ability to measure small otoliths***
729 The smallest pink salmon otolith measured here was 2.5 mg, which is the smallest
730 otolith for which $\delta^{15}N_{oto}$ has been measured to our knowledge. Relative to previous
731 methods that required much more otolith material for analysis, the ability to measure
732 small otoliths transforms $\delta^{15}N_{oto}$ into a tool that can be used for individual fish. Otolith N
733 content in this study ranged from 15.8 nmols mg^{-1} for wild cod to 21.3 nmols mg^{-1} for
734 farmed cod, and the concentration for intact, uncrushed otoliths is higher (32.0 nmols mg^{-1}
735 for intact pink salmon otoliths). With the current N blank of 0.3 – 1 nmol for $\delta^{15}N_{oto}$,
736 and with the goal of minimizing N blank contribution to < 5 % of overall N, this means
737 that as little as 0.4 mg of material or as little as 0.2 mg of material for the most OM-poor
738 and OM-rich species in this study, respectively, are required for analysis. Intra-otolith
739 microsampling may be able to resolve $\delta^{15}N_{oto}$ information from different time periods of
740 a fish's life. For example, juvenile and adult $\delta^{15}N$ from the same fish could be compared
741 by measuring the nucleus and the outer edge of the same otolith, an archival record that is

742 not available from other tissues. Application of $\delta^{15}N_{oto}$ is therefore not constrained to fish
743 with large otoliths, does not require multiple otoliths in order to make a single
744 measurement, and may allow for multiple measurements within individual otoliths.
745
746 ***6.5 Variation in $\delta^{15}N$ among individual fish***
747 Two factors, baseline (primary producer) $\delta^{15}N$ and diet (e.g., trophic level),
748 control the $\delta^{15}N$ of metazoans. Pink salmon from the same cohort have highly similar life
749 history patterns (Bonar et al., 1989) and thus individuals from the same cohort experience
750 a high degree of similarity in baseline $\delta^{15}N$ over their lifetimes. Thus, in the present
751 study, $\delta^{15}N_{oto}$ variations among pink salmon otoliths studied likely reflect differences
752 diet. Otolith size is a proxy for fish size under most conditions when fish are the same age
753 (Templeman and Squires, 1956; but see Mosegaard et al., 1988; Wright et al., 1990;
754 Barber and Jenkins, 2001 for exceptions relating to decoupling between fish growth and
755 otolith size). Thus, the positive relationship between $\delta^{15}N_{oto}$ and otolith size (Fig. 9) is
756 consistent with a correlation between fish size and diet, with larger fish having higher
757 effective trophic level. This was consistent with our expectations, as this species
758 undergoes an ontogenetic dietary shift (Aydin et al., 2005), and larger pink salmon are
759 capable of consuming larger prey (Aydin et al., 2005; Cross et al., 2005). Additionally,
760 quality prey is linked to higher growth rate for pink salmon in the northern Gulf of
761 Alaska and Prince William Sound (Aydin et al., 2005; Cross et al., 2005). The correlation
762 is not driven by fish age: returning pink salmon are two years old in this region (Bonar et
763 al., 1989). For some species, a higher $\delta^{15}N_{oto}$ at greater otolith mass may not necessarily

764 correspond to higher trophic level. For example, Choy et al. (2012) used amino acid-
765 specific $\delta^{15}N$ and found that variability in bulk muscle tissue $\delta^{15}N$ of lanternfishes and
766 dragonfishes resulted from variation in baseline $\delta^{15}N$, as opposed to trophic level.
767 However, baseline $\delta^{15}N$ has no known reason to covary with the size of otolith (or fish) in
768 pink salmon caught in Prince William Sound. Thus, in this case, the higher trophic level
769 of larger fish within the cohort likely explains the higher $\delta^{15}N_{oto}$ of larger otoliths.
770 As otolith growth increases volumetrically, each new layer of aragonite is
771 volumetrically greater than the previous layer (Anderson et al., 1992). Thus, $\delta^{15}N_{oto}$ is
772 weighted toward the $\delta^{15}N$ of the most recent diet (assuming that N content is constant
773 among consecutive layers, which is true at least for the fish examined here). Thus, $\delta^{15}N$
774 in later life is disproportionately important. For pink salmon in the northern Gulf of
775 Alaska, this effect is likely compounded by the fact that the ontogenetic shift to higher
776 trophic level (and thus higher-$\delta^{15}N$ prey) is also associated with faster growth due to the
777 higher nutritional quality of the high trophic level diet (Aydin et al., 2005).
778 Similar to pink salmon for which baseline was similar for all individuals in the
779 same cohort, both species of farmed trout were reared in adjacent freshwater raceways,
780 thus controlling for the $\delta^{15}N$ of their diet. Since farmed trout and wild pink salmon
781 encountered different diet $\delta^{15}N$, direct comparison of $\delta^{15}N_{oto}$ is not ecologically relevant.
782 As farmed brown trout and rainbow trout consume a formulated fish feed for their post-
783 larval diet, as opposed to wild prey, and consumed this food for their entire life history,
784 we hypothesized that differences in otolith (and thus fish) size would not be correlated to
785 changes in $\delta^{15}N_{oto}$, and, indeed, we found no relationship between otolith size and $\delta^{15}N_{oto}$

786 for either species (Fig. A5). This observation serves as a negative control to confirm that
787 $\delta^{15}N_{oto}$ is a robust recorder of the $\delta^{15}N$ of a fish's diet.
788
789 ***6.6 Variation in $\delta^{15}N_{oto}$ within groups of wild and farmed fish***
790 The standard deviation of $\delta^{15}N_{oto}$ was similar for a wild pink salmon cohort and
791 two farm-reared trout cohorts (0.4‰, 0.3‰ and 0.4‰ respectively). It is surprising that
792 the standard deviation for the wild population is not greater than for farmed fish.
793 However, as pink salmon are usually two years old during their return migration and
794 were harvested in the same geographic area, the pink salmon used in this study share a
795 highly similar life history. Other species with more variable life history traits may have
796 greater among-individual variability, e.g., as found for age-2 GB cod (1σ = 0.7 ‰; Fig.
797 11). The variability in $\delta^{15}N_{oto}$ within a group of fish must be determined on a case-by-
798 case basis. Nonetheless, the highly-conserved $\delta^{15}N_{oto}$ in wild pink salmon demonstrates
799 the cohort-level fidelity of $\delta^{15}N_{oto}$ of at least some wild fish. Lastly, the low standard
800 deviation in $\delta^{15}N_{oto}$ among farm-reared individuals implies that physiologically-induced
801 variability is not a significant contributor to the signal in otoliths, indicating that $\delta^{15}N_{oto}$
802 of an entire population may be well represented by a relatively small number of $\delta^{15}N_{oto}$
803 measurements.
804
805 ***6.7 Variation in N content between wild and farmed Atlantic cod***
806 In comparing farmed versus wild Atlantic cod in the Gulf of Maine, we found that
807 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 $\delta^{15}N_{oto}$ offset compared to $\delta^{15}N_{wmt.}$ Additionally, brown trout and rainbow trout fed the same food had indistinguishable $\delta^{15}N_{oto}$ despite the different N contents. This implies that diet plays a dominant role in setting $\delta^{15}N_{oto}$.

6.8 Comparison of $\delta^{15}N_{wmt}$ to $\delta^{15}N_{oto}$

In wild and farmed Atlantic cod, as expected, muscle and otolith $\delta^{15}N$ 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 line, and (2) the much lower $\delta^{15}N_{oto}$ compared to $\delta^{15}N_{wmt}$. First, fish muscle turns over on timescales of months to years, depending on metabolism and other factors (e.g., Logan et al., 2006; Ankjærø 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 $\delta^{15}N_{oto}$ is from the fish's early life whereas $\delta^{15}N_{wmt}$ records recent $\delta^{15}N$, 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 $\delta^{15}N_{oto}$ compared to $\delta^{15}N_{wmt}$ was first reported by Grønkjær et al. (2013), who also measured Atlantic cod otoliths. Grønkjær et al. (2013) found that cod otolith $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N_{oto}$ compared to $\delta^{15}N_{wmt}$ for this species. Moreover, there is no *de facto* reason that otolith $\delta^{15}N$ and muscle $\delta^{15}N$ 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 for different tissue types within the same fish (Macneil et al., 2005; Logan et al., 2006; Buchheister and Latour, 2010). Tissue-specific patterns in nitrogen 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 $\delta^{15}N$—diet, baseline, and metabolism—also affect otolith $\delta^{15}N$. This demonstrates the suitability of $\delta^{15}N_{oto}$ for investigating the same types of ecological questions as $\delta^{15}N_{wmt}$.

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

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

852 the end of their lives is predicted to be 3.5 ‰ higher than modern cod (18.6 ‰ compared
853 to 15.1 ‰) from the same region. This is calculated based on the regression $\delta^{15}N_{oto}$ =
854 0.69 (± 0.33 95% confidence interval) * $\delta^{15}N_{wmt}$ - 2.74 (± 4.78 95% confidence interval).
855 We interpret the $\delta^{15}N_{wmt}$ with caution because of the low sample size, and also because
856 no otoliths from similarly large modern fish were available for comparison with the
857 otoliths from ~meter-long cod in the midden. Nevertheless, the finding that fossil cod
858 $\delta^{15}N_{oto}$ is 2.2‰ higher than the modern value calls for interpretation.
859 Two possible causes for change in cod trophic level (and thus $\delta^{15}N_{oto}$) can be
860 identified. First, cod trophic level tends to rise with fish size, as fish size in itself changes
861 the prey items that can be consumed by gape-limited predators such as cod. Second,
862 environmental and ecological changes can cause a change in the $\delta^{15}N$ of the prey
863 available to cod even without a change in the size of the cod (i.e. "trophic-level-at-size").
864 We address these in turn.
865
866 ***6.9.1. Role of fish size***
867 Cod are known to become increasingly piscivorous (fish-consuming) as they
868 grow, including increasingly cannibalistic, based on stomach content data (Bigelow and
869 Schroeder, 1953; Pálsson, 1983; Link and Garrison, 2002b; Smith et al., 2007; Pálsson
870 and Björnsson, 2011). In the Gulf of Maine, stomach content data spanning 1973-1998
871 indicate that small cod (31-40 cm) consumed mostly crustaceans (an average of 54% by
872 volume) and a smaller contribution of fish (18%) whereas the diet of large cod (81-90
873 cm) contained predominantly fish (66%) (Link and Garrison, 2002b). For cod in the

874 largest size range (> 120 cm), diet also included significant contributions from bluefish,
875 goosefish, and redfish, in addition to the fish species listed above (for a total of 83% fish)
876 (Link and Garrison, 2002b). The increasing percentage of fish prey, including shifts in
877 species with increasing cod size, suggest that a size-related increase in cod $\delta^{15}N$ is very
878 likely. Since fish prey usually have higher $\delta^{15}N$ than macroinvertebrate prey in this
879 region (e.g., Fry, 1988; but see Sherwood and Rose, 2005, for examples in which pelagic
880 and benthic prey can have overlapping $\delta^{15}N$ signatures due to differing baselines), large
881 cod would be expected to have higher $\delta^{15}N$ than small cod due to a higher proportion of
882 fish in the diet of large cod. The specific relationship between cod size and $\delta^{15}N$ has not
883 been investigated in the Gulf of Maine to our knowledge. However, Jennings,
884 Greenstreet, et al. (2002) find that Atlantic cod in the North Sea increase by 3 ‰ from 40
885 to 140 cm. Other piscivorous fish species, which are known based on stomach contents to
886 shift from lower trophic level prey to higher trophic level prey, undergo a 1 to 4‰
887 allometric increase in $\delta^{15}N$ between intermediate and large lengths (e.g., Hobson and
888 Welch, 1995; Jennings et al., 2002; Graham et al., 2007; Wells et al., 2008; Christiansen
889 and Hop, 2012; Glaz et al., 2012; Kim et al., 2012; Ramsvatn and Pedersen, 2012; Weng
890 and Lee, 2015).The $\delta^{15}N$ of Arctic char, a similarly piscivorous (and cannibalistic)
891 species as cod, increases by 3.7‰ from intermediate lengths to large lengths (i.e., from
892 30 cm to 50 cm; Hobson and Welch, 1995). In the present study, the largest midden
893 otolith is estimated to have come from a 97 cm cod whereas the largest modern otolith
894 came from a 78 cm cod. (The finding that historical cod were larger is not surprising, as
895 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 $\delta^{15}N_{oto}$ 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 $\delta^{15}N_{oto}$ in the Gulf of Maine.

Environmental and ecological change may also have contributed to the apparent change in cod $\delta^{15}N$. 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 $\delta^{15}N$-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; Link and Garrison, 2002b; 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 $\delta^{15}N$ is consistent with changing prey patterns in addition to the changes in cod size. Potential changes in $\delta^{15}N$-at-size, which may reflect large scale environmental and ecological change, will be elucidated in future work.

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

Possible approaches to address baseline changes include the following: (1)

comparing $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 for $\delta^{15}N$ and $\delta^{13}C$ in white muscle tissue. McMahon, Fogel, et al. (2011) introduced a method by which amino acid-specific $\delta^{13}C$ in otolith OM was analyzed and applied to retrospectively determine nursery grounds and migratory patterns of snapper in the Red Sea (McMahon, Fogel, et al., 2011; McMahon et al., 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 $\delta^{15}N_{oto}$ analysis. Method development and ground-truthing may address these issues in the future.

Here, for explanatory purposes, we consider possible influences of baseline $\delta^{15}N$ change on the observed change in cod $\delta^{15}N_{oto}$. The dominant driver of "baseline" $\delta^{15}N$ variation in the open ocean is the $\delta^{15}N$ of nitrate assimilated into biomass in surface waters. $\delta^{15}N$ of this assimilated nitrate is controlled by both the $\delta^{15}N$ of the nitrate supply and the degree of nitrate consumption in surface waters. In the Gulf of Maine coastal

940 region, the degree of consumption is usually complete over the course of the
941 spring/summer growth period, which would tend to make the $\delta^{15}N$ of the nitrate supply
942 the dominant driver of baseline change.
943 In the case of the North Atlantic there are regional variations in the $\delta^{15}N$ of the
944 nitrate supply to the euphotic zone, with the lowest $\delta^{15}N$ occurring in the subtropical gyre
945 (Knapp et al., 2008). Ocean circulation changes might alter the $\delta^{15}N$ of the nitrate supply
946 to the Gulf of Maine by changing the relative importance of this low $\delta^{15}N$ subtropical
947 nitrate relative to the nitrate imported to the surface at higher latitudes, and a $\delta^{15}N$ change
948 in soft coral has been interpreted in this way (Sherwood et al., 2011). If this process were
949 important in our cod $\delta^{15}N_{oto}$ decline, it would require a greater relative input of this
950 subtropical nitrate to the Gulf of Maine under modern conditions. Existing hydrographic
951 data do not show an obvious signature of this process (Townsend et al., 2010; Townsend
952 et al., 2015; Feng et al., 2016), but it cannot be ruled out.
953 Anthropogenic impacts on the $\delta^{15}N$ of fixed N supply to the Gulf of Maine
954 euphotic zone must also be considered. Rivers in the present tend to deliver biologically
955 available N with a high $\delta^{15}N$, leading to increases of up to 7 ‰ in macroalgae
956 (McClelland et al., 1997; Savage, 2005) and increases of up to 4 ‰ for primary
957 consumers and fish near rivers or wastewater point sources (Fry, 1999; Pruell et al., 2006;
958 Corbett et al., 2015; Duprey et al., 2017). Explaining the decline in modern $\delta^{15}N_{oto}$ would
959 require that this high $\delta^{15}N$ source is less important than in the 17th century, which seems
960 unlikely. Moreover, several studies report that dilution with seawater reduces the $\delta^{15}N$
961 impacts from river- or wastewater-delivered waters within only 1 to 30 km from the point

962 source (Savage, 2005; Pruell et al., 2006; Corbett et al., 2015; Duprey et al., 2017),
963 arguing against a role in cod $\delta^{15}N_{oto}$ changes in general. Lastly, atmospheric N
964 deposition, which has a low $\delta^{15}N$, typically constitutes a low contribution in nutrient-rich
965 coastal and shelf systems. In summary, while baseline effects cannot be precluded with
966 the existing data, a trophic effect from the decrease in fish size and/or a change in trophic
967 level-at-size currently represent our best explanations for the apparent Gulf of Maine cod
968 $\delta^{15}N_{oto}$ decline since the 17th Century.
969
970 ***6.10 Future applications of $\delta^{15}N_{oto}$***
971 Analyzing otoliths from 4000-year-old midden mounds or historical sites (Harris,
972 2011; Limburg et al., 2011), 9000-year old shelf sediments (Elder et al., 1996), or 33.7
973 Myr shelf sediments (Ivany et al., 2000) would allow for reconstructing pre-disturbance
974 ecological conditions. Other than species interactions captured in the fossil record and the
975 physiology of ancient organisms indicating dietary preferences, there are few options for
976 investigating the ecology of ancient oceans. Whether prehistoric otoliths still retain OM
977 is as yet unknown; however, recent confirmation of protein in dinosaur bones (Schroeter
978 et al., 2017) suggests that trace amounts of OM may be present in some ancient fossil
979 otoliths. Many paleo-ecological studies use otoliths to reconstruct the species diversity or
980 paleoecology of past oceans (Frizzell and Dante, 1965; Aguilera and Rodrigues de
981 Aguilera, 2001; Schwarzhans et al., 2016), and some of these otoliths are exceptionally
982 well preserved (Gierl et al., 2013). Possible studies with policy implications include
983 examining changes in food webs resulting from climate change or from the arrival of

984 European and American commercial fishing activities in the Western Atlantic. As
985 described above, analyses of co-occurring fossils, especially of primary consumers such
986 as bivalves or gastropods found in the same strata as otolith samples, might help to
987 constrain baseline $\delta^{15}N$ for each time period.

988 $\delta^{15}N_{oto}$ has potential for investigating long term ecological patterns in populations
989 on decadal and centennial time scales (e.g., Rowell et al., 2010; Sirot et al., 2017). Many
990 countries with fisheries economies have otolith archives spanning the 20th century, due
991 to long term government sampling programs for collecting biological data on fish
992 populations, which often include the collection of otoliths for fish age determination.
993 Pairing $\delta^{15}N_{oto}$ with stock indices, e.g., identifying dietary changes in spawning stocks
994 leading up to recruitment of especially large year classes, is an intriguing possible
995 application of this method.

996 Combining $\delta^{15}N_{oto}$ methods with other otolith chemical measurements would
997 enhance the utility of each for investigating fisheries ecology. Otolith microchemistry is
998 used widely to investigate migratory behavior, habitat residency, and population
999 connectivity of wild fishes (Campana and Thorrold, 2001; Sturrock et al., 2012). $\delta^{15}N_{oto}$
1000 can provide an ecological dimension due to the dependence of $\delta^{15}N_{oto}$ on diet and
1001 baseline. For example, migratory versus resident subpopulations (e.g., as reviewed by
1002 Secor, 2015) are likely to exhibit different $\delta^{15}N_{oto}$, whether due to dietary differences or
1003 baseline differences. Other examples are the use of $^{87/86}Sr$ to determine natal stream for
1004 Atlantic salmon (Kennedy et al., 1997; Kennedy et al., 2000; Barnett-Johnson et al.,
1005 2008) in freshwater systems, and the use of element-to-calcium ratios, $\delta^{13}C$ and $\delta^{18}O$ in

1006 the otolith aragonite to determine nursery ground in the marine environment (e.g., Kerr et
1007 al., 2007; Wells et al., 2012; Rooker et al., 2016), and the use of otolith chemistry
1008 measurements to identify sub-population structure of fish populations otherwise known
1009 be homogenous based on genetics (Svedäng et al., 2010). Combining natal stream or
1010 nursery ground identity with $\delta^{15}N_{oto}$ would provide insights concerning whether fish
1011 trophic level, as a result of diet, influences why specific streams or nursery grounds are
1012 more or less productive (resulting in differential recruitment success). Dietary
1013 reconstruction using $\delta^{15}N_{oto}$ has great potential to provide ecological mechanisms for fish
1014 behavior when paired with the geographic or migratory information from otoliths.
1015 Lastly, future optimization of micromilling with $\delta^{15}N_{oto}$ will be useful for
1016 ecological investigations in both the modern and past ocean. As many species undergo
1017 ontogenetic changes in diet or habitat, micromilling would provide many exciting
1018 applications for reconstructing fish behavior and resource use. Analysis of early life
1019 history (otolith core) material may allow for tracking long term changes in nitrogen
1020 cycling at the base of the marine food chain, as juvenile fishes consume primary
1021 consumers such as copepods that integrate isotopic changes in baseline. The current
1022 minimum analytical requirement for this method is ~6 nanomoles, corresponding to ~0.4
1023 mg of material for the most OM-poor species measured in the current study. This roughly
1024 equates to the ability to micromill otolith core and outer edge, with up to two time points
1025 in between depending on the species and the size of the otolith. While coarse resolution
1026 relative to $\delta^{18}O$ or laser ablation-based element:calcium measurements, the resulting
1027 ontogenetic information may be enlightening.

7. CONCLUSION

A two-step cleaning process (surficial 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 data. 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 $\delta^{15}N$ 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 $\delta^{15}N_{oto}$ 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

$\delta^{15}N_{oto}$ for 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.

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FIGURE CAPTIONS

Table 1: **Sample locations and dates.**

Table 2: **Tests investigating the analytical precision, ecological accuracy, and preservation of $\delta^{15}N_{oto}$.**

Table 3: **Effects of cleaning reagent on three species.** $\delta^{15}N_{oto}$ and N content of three otolith standards, cod otolith standard (CDS), queen snapper standard (QSN), and pink salmon standard (PSS) for two cleaning treatments. Differences in N content between cleaning treatments are thought to stem from coupled dissolution- reprecipitation in the autoclave step required for POR cleaning.

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. 6). Yellow arrows show the sulcus acusticus, a grooved feature exhibited by all fish otoliths (highlighted here to demonstrate that it is a naturally occurring feature of the otolith and not an artifact from preservation or cleaning).

Fig. 2. **Diagram of cleaning methods for fossil otoliths.** The $\delta^{15}N_{oto}$ 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 $\delta^{15}N_{oto}$ 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.

Fig. 3. **Analytical precision of the method for in-house standards.** The 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 sample batch run on the GC-IRMS. The inter-batch mean for cod otolith standard (CDS) is 6.9 ± 0.3 ‰ and the N content is 15.8 ± 1.8 nmols N mg^{-1} (11% percent standard deviation) across eight sample batches. Pink salmon otolith standard (PSS) is 14.4 ± 0.3‰ and the N content is 17.1 ± 2.0 nmols N mg^{-1} (12% percent standard deviation) N content across eleven sample batches.

Fig. 4. **Time course of cod otolith standard (CDS) exposure to sodium hypochlorite.** Samples were either heated using a water bath (60ºC) or maintained at room temperature (22ºC). Despite declining N content over the first 6 hours, $\delta^{15}N_{oto}$ was similar across all time points within each treatment. N content 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, $p = 0.36$).

Fig. 5. **Effect of grain size and cleaning reagent on of $\delta^{15}N_{oto}$ and N content.** Boxplots showing interquartile range of $\delta^{15}N_{oto}$ (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 $\delta^{15}N_{oto}$ and N content at larger grain sizes, the $\delta^{15}N_{oto}$ 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; $p = 0.05$).

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 (as per Fig. 2). Otolith A (square symbols) was a larger otolith (similar to Fig. 1c) and Otolith B (triangle symbols) was a smaller otolith (similar to Fig. 1d). After grain cleaning, Otolith A mean $\delta^{15}N_{oto}$ changed from 10.3 ± 0.2‰ to 9.5 ± 0.3‰ (Welch's *t*-test, $p < 0.05$). For Otolith B, mean $\delta^{15}N_{oto}$ did not differ between surface only and surface and grain cleaning (7.9 ± 0.2‰ compared to 7.5 ± 0.4‰; Welch's *t*-test, $p = 0.11$). N content decreased from 19.8 ± 1.6 to 15.8 ± 0.7 nmol N mg^{-1} for Otolith A (Welch's *t*-test, $p < 0.05$); while N content remained unchanged from 14.3 ± 0.3 to 14.6 ± 0.5 nmol N mg^{-1}, for Otolith B (Welch's *t*-test, $p = 0.35$).

Fig. 7. **Cohort-level variability in $\delta^{15}N_{oto}$.** Intra-cohort variability of farmed brown trout, farmed rainbow trout, and wild pink salmon (11.5 ± 0.3‰, 11.3 ± 0.4‰, 14.5 ± 0.4‰ 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.

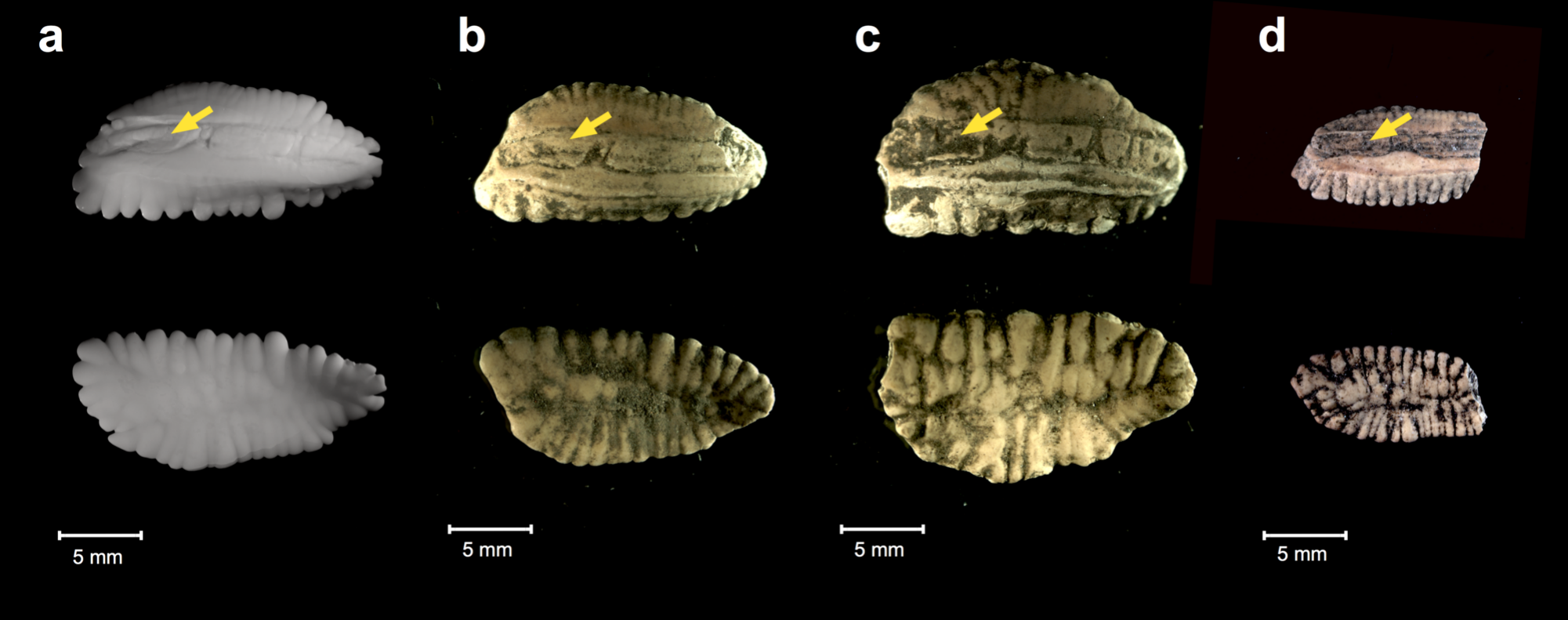
Fig. 8. **Left versus right $\delta^{15}N_{oto}$.** Left (L) versus right (R) otolith for brown trout (blue symbols), rainbow trout (purple symbols), and pink salmon (gray and empty symbols) showing greater variability among individual fish than between L vs. R otoliths from the same fish. Intact otolith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

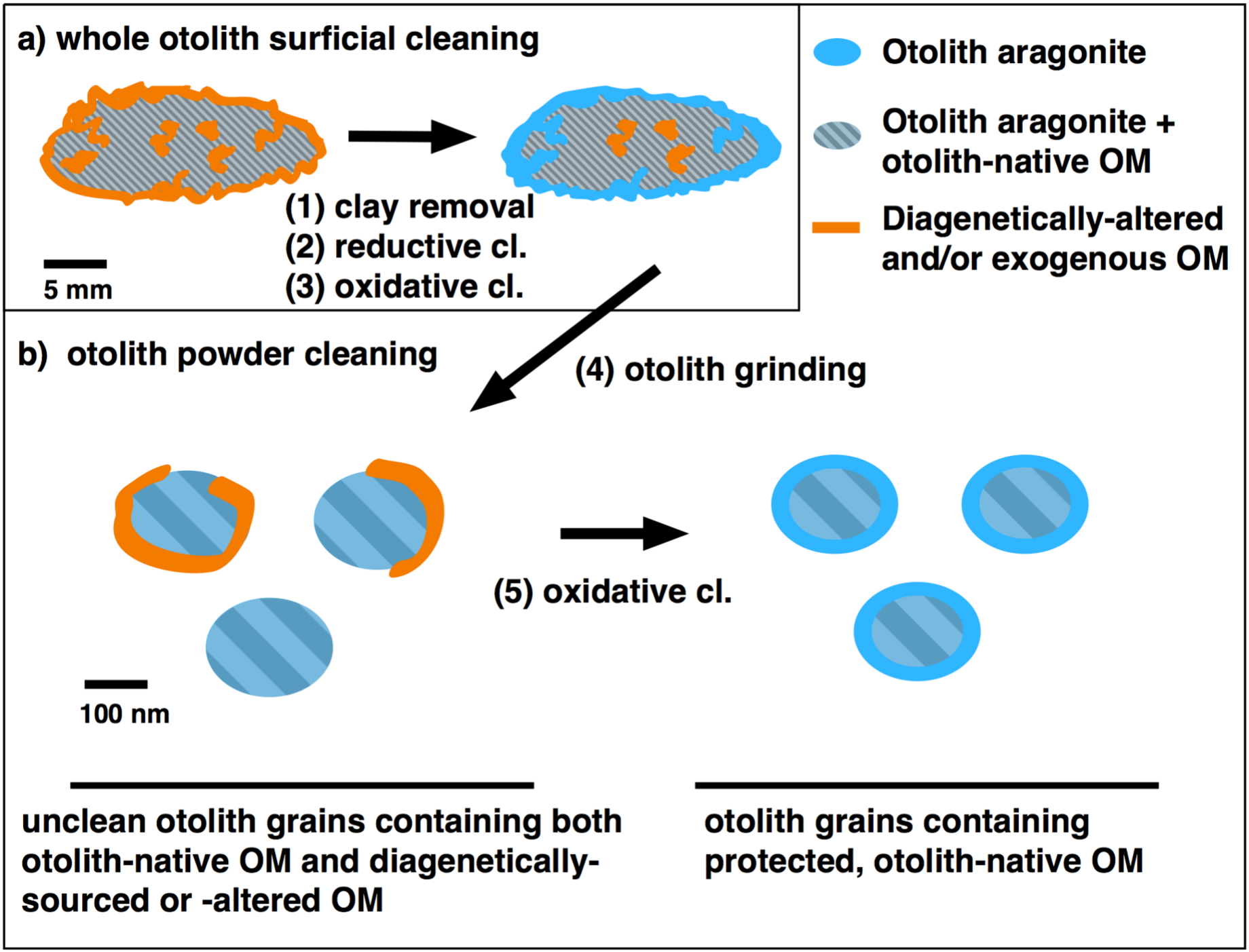
Fig. 9. **Otolith mass vs. $\delta^{15}N_{oto}$ for wild pink salmon.** Otolith mass (a proxy for fish size) versus $\delta^{15}N_{oto}$ for individual wild pink salmon showing a positive relationship between fish size and fish $\delta^{15}N_{oto}$ of intact otoliths even within a small range of $\delta^{15}N$ (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 $\delta^{15}N_{oto}$ 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.

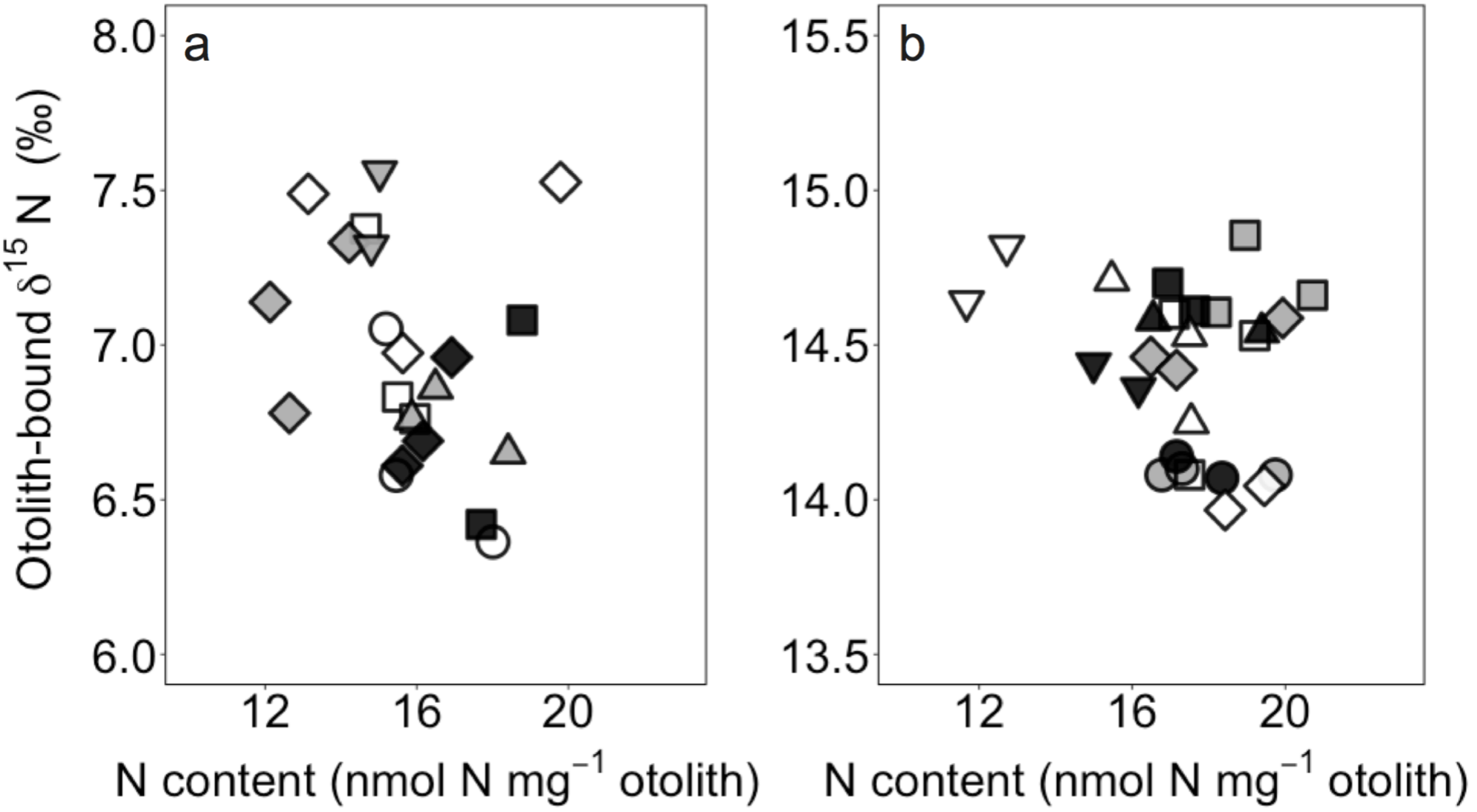
Fig. 10. **Paired measurements of $\delta^{15}N_{wmt}$ vs. $\delta^{15}N_{oto}$ for Atlantic cod.** Paired otolith and white muscle $\delta^{15}N$ for farmed (open triangles) and wild (gray triangles) Atlantic cod. Dashed line shows the least squares regression between $\delta^{15}N_{wmt}$ and $\delta^{15}N_{oto}$ ($y = 0.69x - 2.74$; $r^2 = 0.64$). Error bars are one standard deviation of replicate subsamples. Mean white muscle $\delta^{15}N$ standard deviation for replicate measurements was 0.04‰.

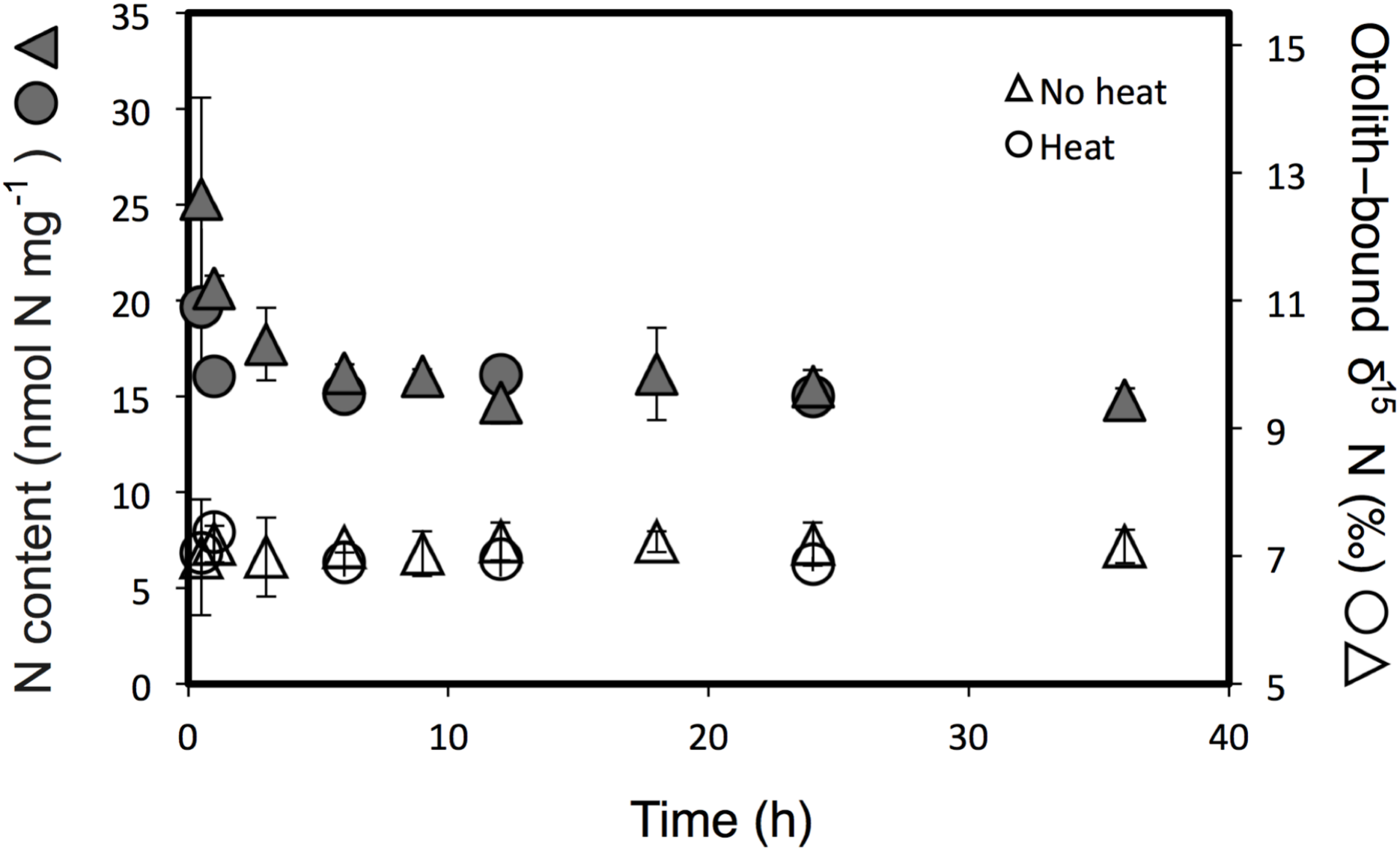
Fig. 11. **$\delta^{15}N_{oto}$ and N content for historical and modern Atlantic cod**. $\delta^{15}N_{oto}$, N content, and otolith mass mean ± 1σ for 17th century Atlantic cod otoliths from a historical fishing station in the western Gulf of Maine (diamond symbol; n = 4 fish), for modern commercially-harvested 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

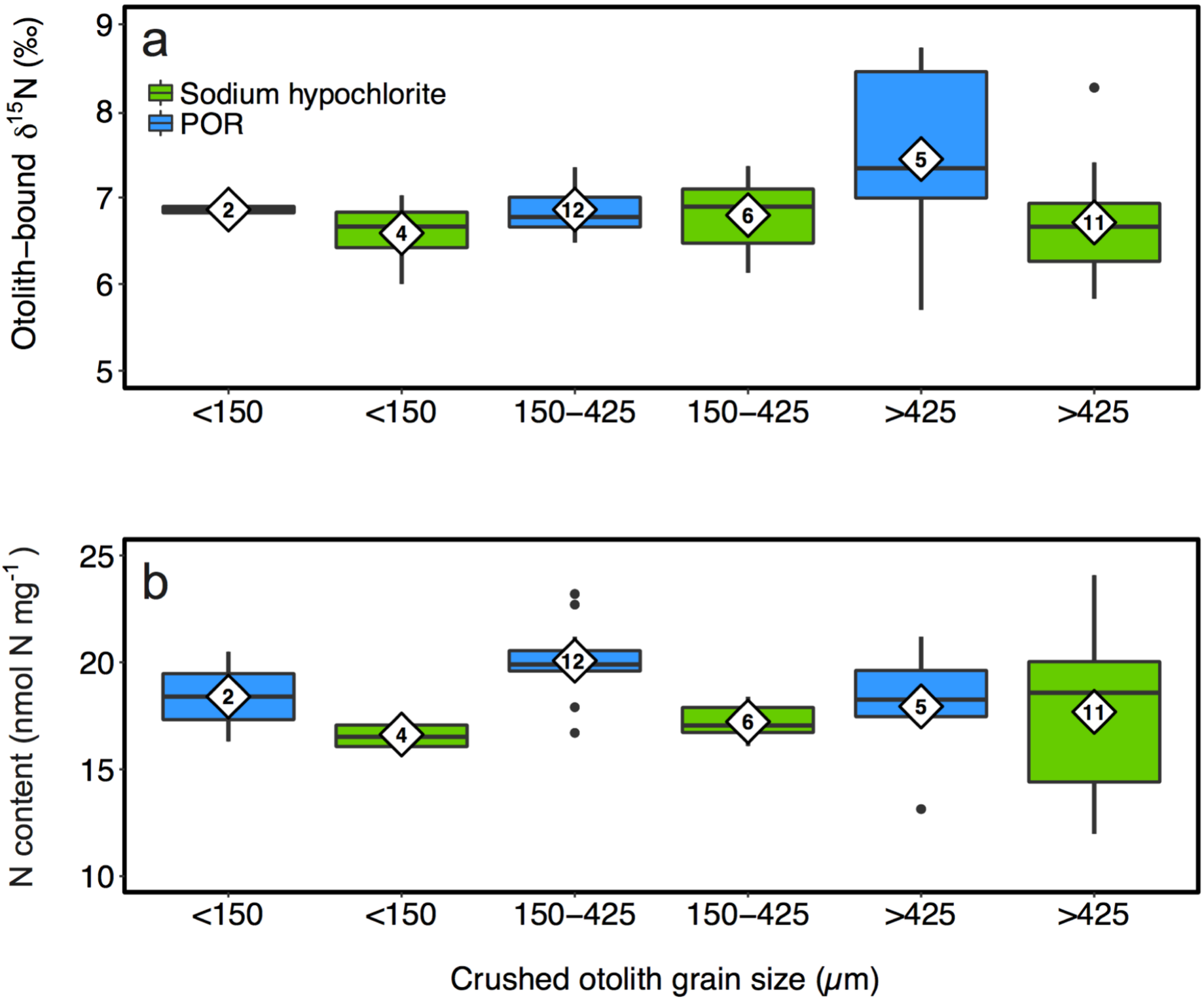
homogenized CDS made of four additional modern Gulf of Maine cod otoliths). The N content for farm-raised Atlantic cod (black arrow; n = 3 fish) is plotted without $\delta^{15}N_{oto}$ because fish were reared on commercial aquafeed not relevant for the comparison to wild fish.

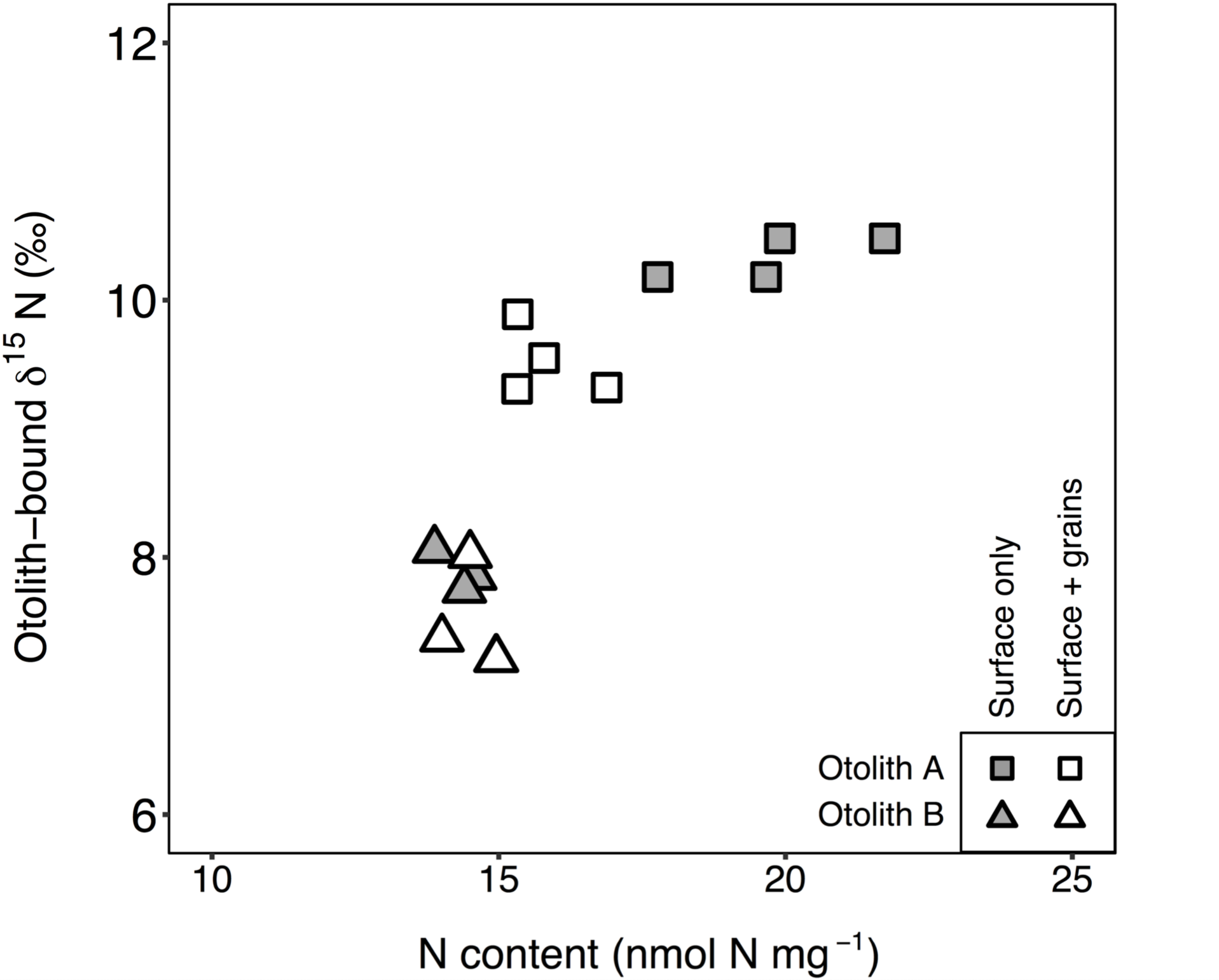

a
b
c
d
5 mm
5 mm
5 mm
5 mm

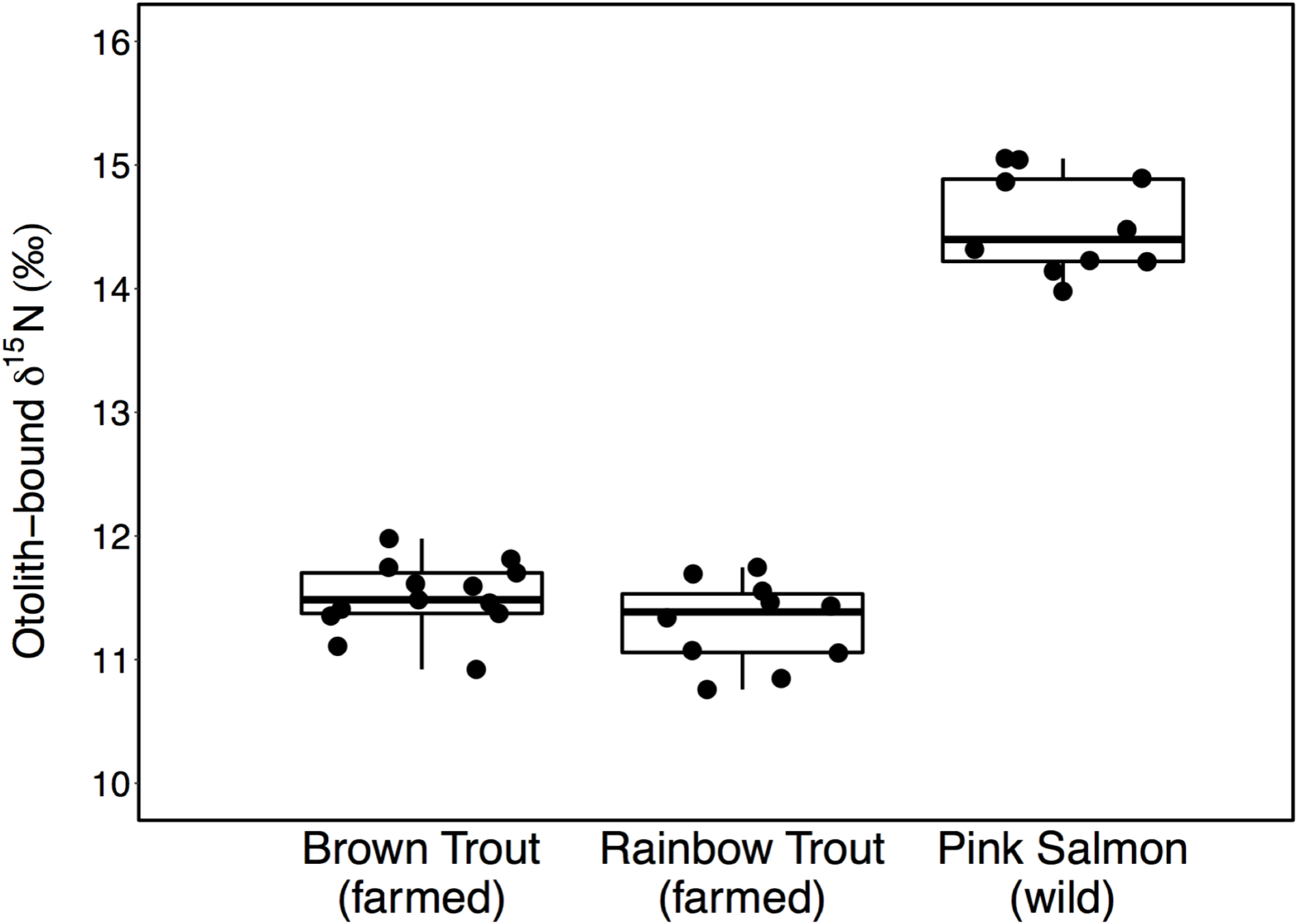

a) whole otolith surficial cleaning
Otolith aragonite
Otolith aragonite + otolith-native OM
Diagenetically-altered and/or exogenous OM
(1) clay removal
(2) reductive cl.
(3) oxidative cl.
5 mm
b) otolith powder cleaning
(4) otolith grinding
(5) oxidative cl.
100 nm
unclean otolith grains containing both otolith-native OM and diagenetically-sourced or -altered OM
otolith grains containing protected, otolith-native OM

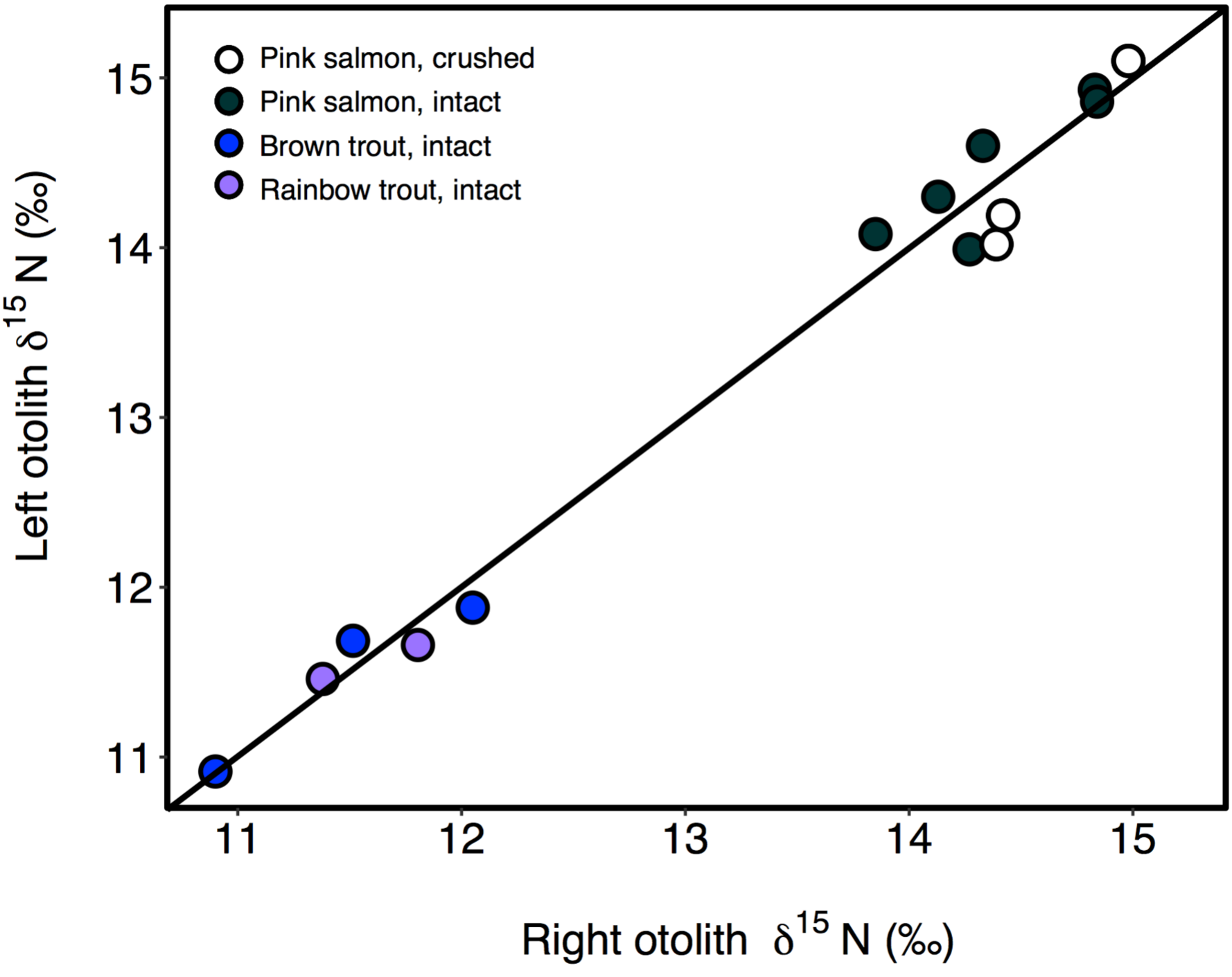

a
b
8.0
7.5
7.0
6.5
6.0
15.5
15.0
14.5
14.0
13.5
12
16
20
Otolith-bound δ15 N (‰)
N content (nmol N mg−1 otolith)
N content (nmol N mg−1 otolith)

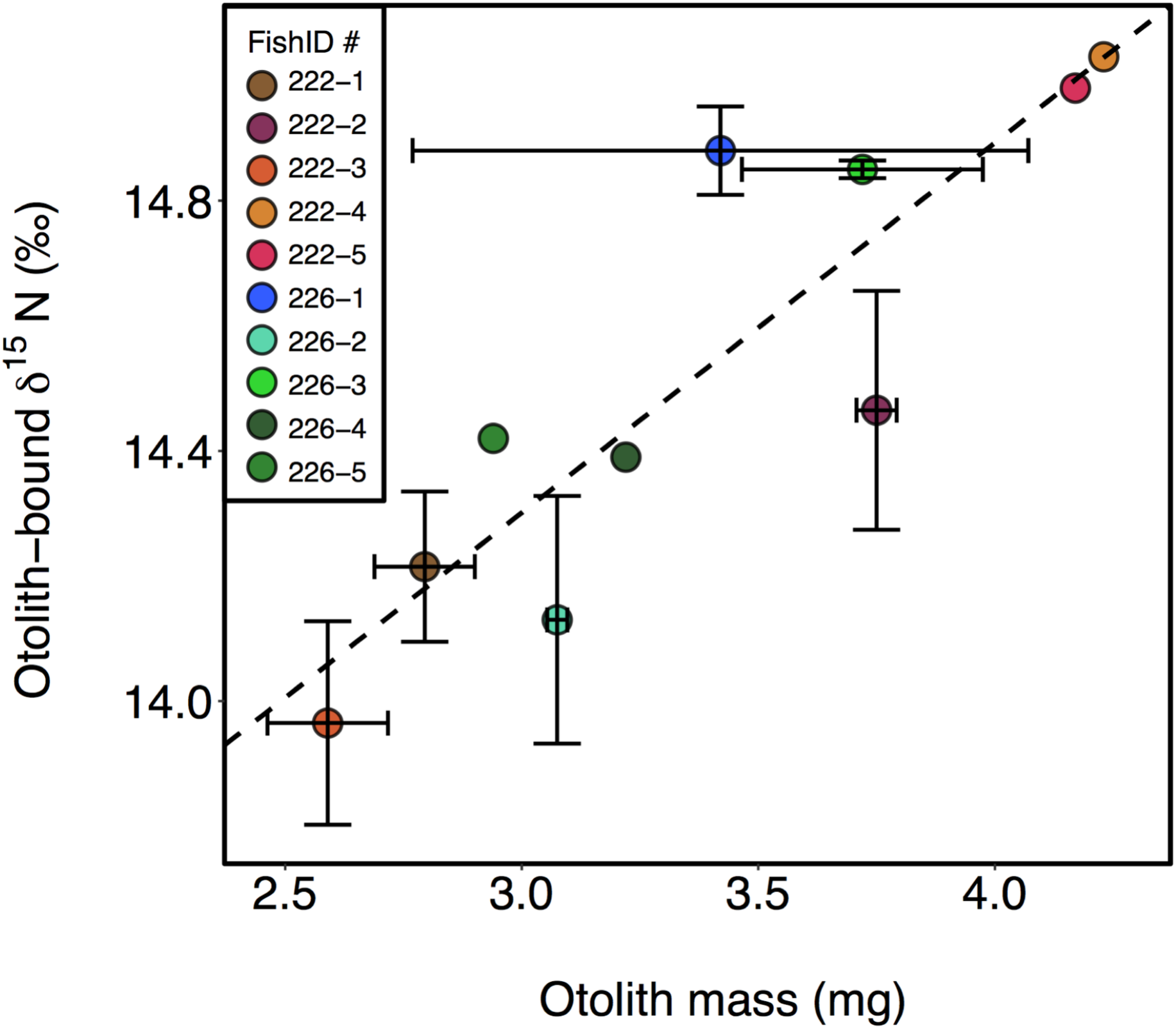

N content (nmol N mg-1) ○ △
Otolith–bound δ15N (‰) ▽ ○ △
No heat
Heat
Time (h)
0
5
10
15
20
25
30
35
0
10
20
30
40
5
7
9
11
13
15

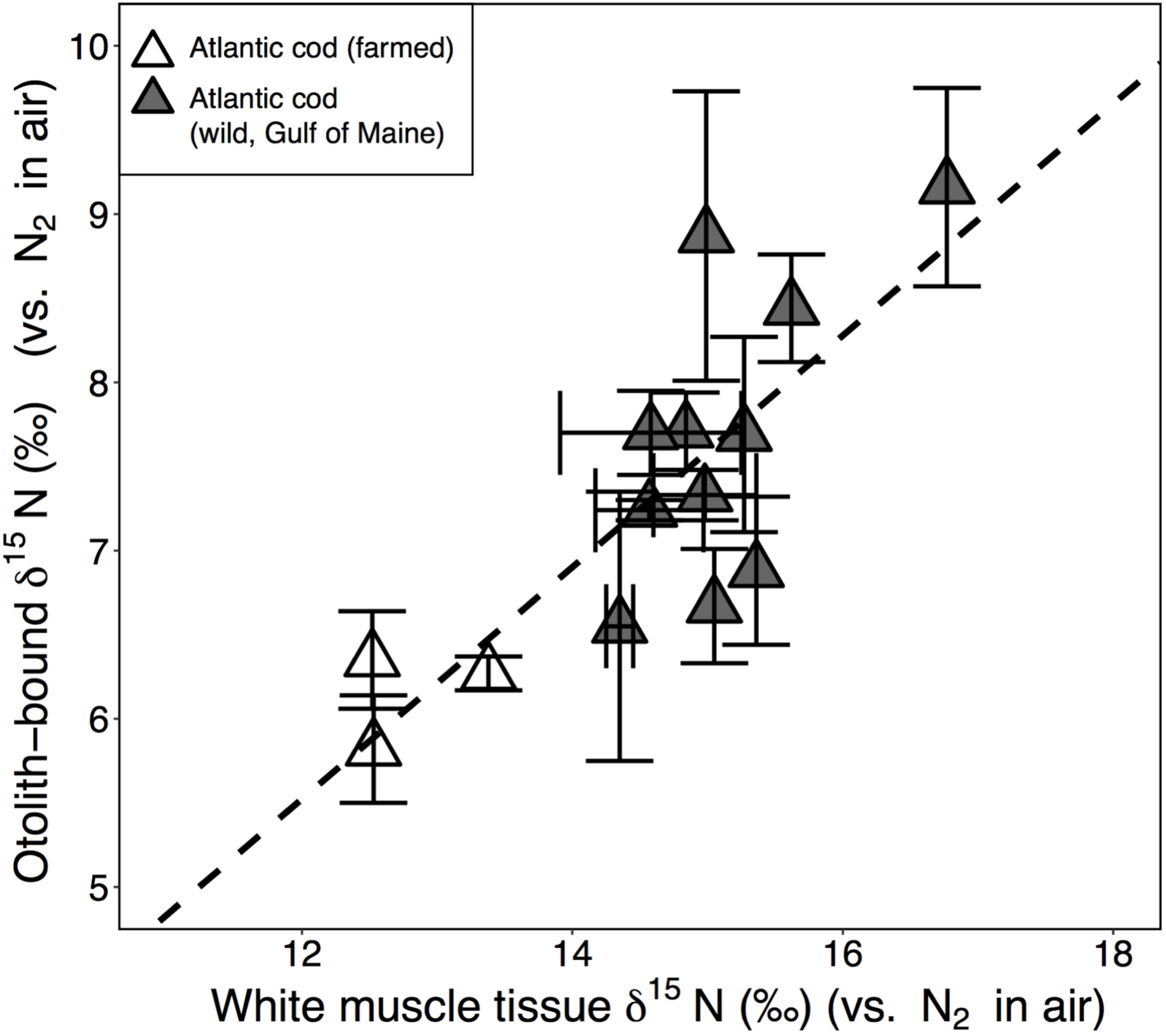

a
Sodium hypochlorite
POR
Otolith–bound δ15N (‰)
9
8
7
6
5
2
4
12
6
5
11
<150
<150
150–425
150–425
>425
>425
b
N content (nmol N mg-1)
25
20
15
10
2
4
12
6
5
11
<150
<150
150–425
150–425
>425
>425
Crushed otolith grain size (μm)


12
10
8
6
10
15
20
25
Otolith–bound δ15 N (‰)
N content (nmol N mg−1)
Surface only
Surface + grains
Otolith A
Otolith B


Otolith–bound δ15N (‰)
16
15
14
13
12
11
10
Brown Trout (farmed)
Rainbow Trout (farmed)
Pink Salmon (wild)


Pink salmon, crushed
Pink salmon, intact
Brown trout, intact
Rainbow trout, intact
15
14
13
12
11
Left otolith δ15 N (‰)
11
12
13
14
15
Right otolith δ15 N (‰)


FishID #
222–1
222–2
222–3
222–4
222–5
226–1
226–2
226–3
226–4
226–5
14.8
14.4
14.0
Otolith–bound δ15 N (‰)
2.5
3.0
3.5
4.0
Otolith mass (mg)


Atlantic cod (farmed)
Atlantic cod
(wild, Gulf of Maine)
10
9
8
7
6
5
12
14
16
18
Otolith–bound δ15 N (‰) (vs. N2 in air)
White muscle tissue δ15 N (‰) (vs. N2 in air)

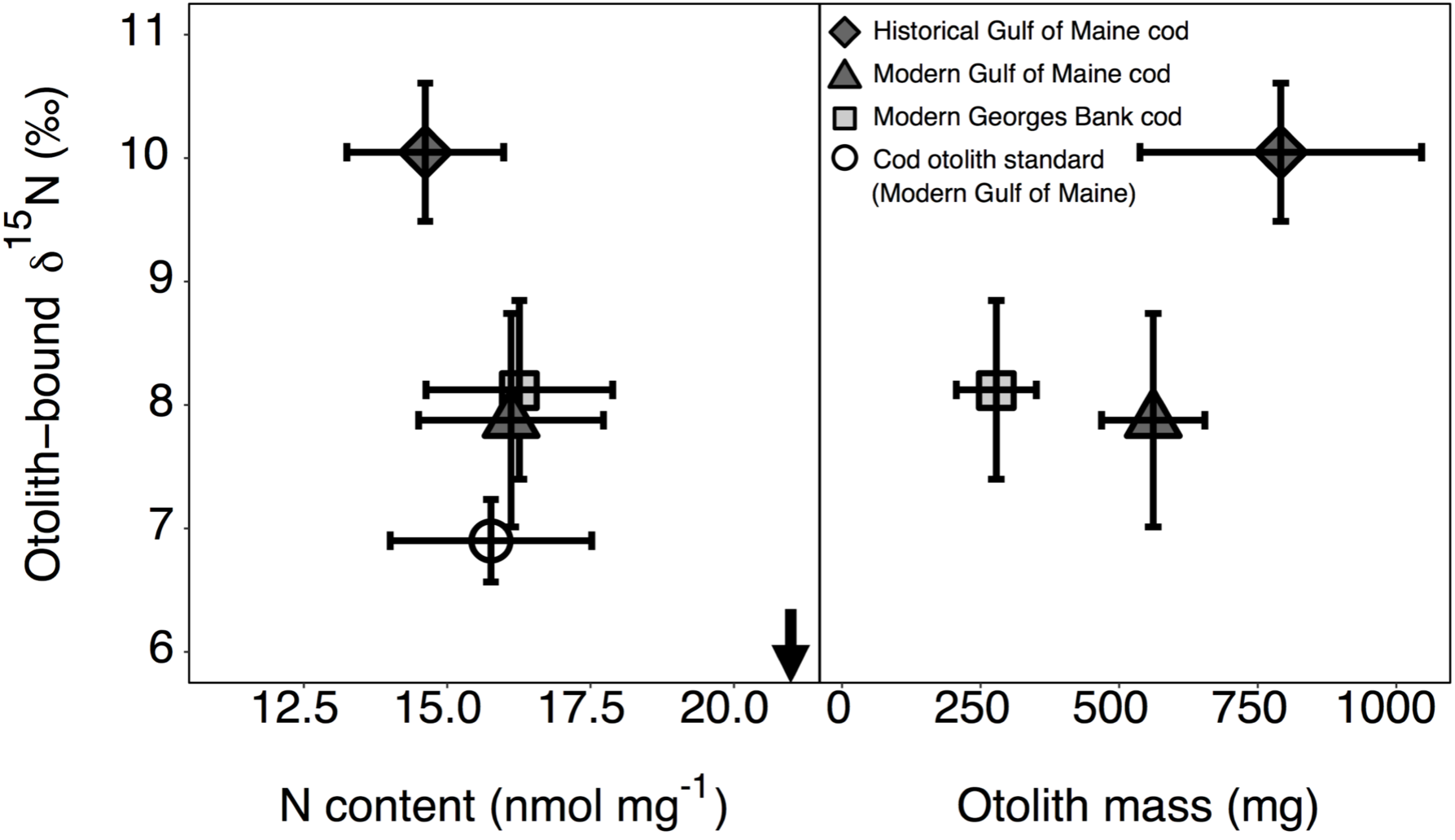

Historical Gulf of Maine cod
Modern Gulf of Maine cod
Modern Georges Bank cod
Cod otolith standard
(Modern Gulf of Maine)
Otolith–bound δ15N (‰)
11
10
9
8
7
6
12.5
15.0
17.5
20.0
0
250
500
750
1000
N content (nmol mg-1)
Otolith mass (mg)

Table 1: Sample locations and dates of otoliths used for $\delta^{15}N_{oto}$

Common name	Latin name	*n*	Origin	Location (of farm, of excavation, of fish landing)	Type	Sampling date
Brown trout	*Salmo trutta*	13	Musky Trout Hatchery	Asbury, NJ	Aquaculture (fish farm)	24 July 2014
Rainbow trout	*Oncorhynchus mykiss*	10	Musky Trout Hatchery	Asbury, NJ	Aquaculture (fish farm)	24 July 2014
Pink salmon	*Oncorhynchus gorbuscha*	10	Alaska Department of Fish & Game, Cordova, AK	Prince William Sound, Alaska	Wild-caught (commercial)	22 August 2008
Atlantic cod (historical)	*Gadus morhua*	4	University of Southern Maine, Cornell University	Smuttynose Island, NH	Wild-caught (commercial)	8 June 2010 (excavation)
Atlantic cod (modern)	*Gadus morhua*	7	Nassau Seafood, Princeton, NJ	Chatham, MA	Wild-caught (commercial)	13 November 2014
Atlantic cod (modern)	*Gadus morhua*	4	Nassau Seafood, Princeton, NJ	Chatham, MA	Cod otolith standard (CDS)	13 November 2014
Atlantic cod (modern)	*Gadus morhua*	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)	*Gadus morhua*	3	University of Maine Center for Cooperative Aquaculture Research (CCAR)	Franklin, ME	Aquaculture (fish farm)	17 March 2015
Pink salmon	*Oncorhynchus gorbuscha*	25	ADF&G, Cordova, AK	Prince William Sound, Alaska	Pink salmon standard (PSS)	22 August 2008
Queen snapper	*Etelis oculatus*	20	Nassau Seafood, Princeton, NJ	Panama City, Panama	Queen snapper standard (QSN)	2 Nov 2014

Table 2: Tests investigating the analytical precision, ecological accuracy, and preservation of $\delta^{15}N_{oto}$.

Analytical[1]	Ecological[2]	Preservation[3]
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 $\delta^{15}N_{oto}$ variability	Exposure time to cleaning 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 $\delta^{15}N_{oto}$	
Intra-fish (L vs. R) comparison	$\delta^{15}N_{oto}$ vs. $\delta^{15}N_{wmt}$	

[1] Experiments investigating the precision of $\delta^{15}N_{oto}$. Refers mostly to experiments conducted with otolith standards.

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

[3] Experiments investigating the degree of resistance of $\delta^{15}N_{oto}$ to diagenesis

Table 3. Effects of cleaning reagent on three species.

		NaOCl	*n*	POR	*n*	*P*
$\delta^{15}N$ *(‰ 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.*