



# In search of the dead zone: Use of otoliths for tracking fish exposure to hypoxia



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## ABSTRACT

Otolith chemistry is often useful for tracking provenance of fishes, as well as examining migration histories. Whereas elements such as strontium and barium correlate well with salinity and temperature, experiments that examine manganese uptake as a function of these parameters have found no such correlation. Instead, dissolved manganese is available as a redox product, and as such, is indicative of low-oxygen conditions. Here we present evidence for that mechanism in a range of habitats from marine to freshwater, across species, and also present ancillary proxies that support the mechanism as well. For example, iodine is redox-sensitive and varies inversely with Mn; and sulfur stable isotope ratios provide evidence of anoxic sulfate reduction in some circumstances. Further, S may be incorporated trophically whereas other elements appear to be taken up directly from water. This research suggests a potential means to identify individual fish exposure to hypoxia, over entire lifetimes. With further testing and understanding, in the future fish may be able to be used as “mobile monitors” of hypoxic conditions.

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## 1. Introduction

Low oxygen or oxygen-free zones are on the increase worldwide as nutrient loads from land to water increase and climate warms (Diaz and Rosenberg, 2008; Keeling et al., 2010; Rabalais et al., 2010). Despite the lethal effects of hypoxia (defined as  $\leq 2 \text{ mL O}_2 \text{ L}^{-1}$ ) and anoxia on many organisms in aquatic ecosystems, obvious impacts on fisheries have been difficult to detect (Breitburg et al., 2009), although adverse impacts have been documented on recruitment (Köster et al., 2005) and fish health (e.g., Thomas and Rahman, 2012; Thomas et al., 2007). Nevertheless, opportunities exist for fish to feed on stressed prey (Long and Seitz, 2008), and adaptation to lower oxygen conditions is documented in some species, notably Atlantic cod *Gadus morhua* in hypoxic North Atlantic and Baltic waters (Andersen et al., 2009) and the bearded goby *Sufflogobius bibarbatus* in anoxic parts of the Benguela ecosystem (Utne-Palm et al., 2010). Thus, there are adaptive advantages for fishes to pursue prey in hypoxic conditions, if they are sufficiently tolerant to spend time foraging (Neuenfeldt et al., 2009; Rahel and Nutzman,

1994), or avoiding predators (Utne-Palm et al., 2010). However, close observation of fish foraging in natural, hypoxic conditions appears to be rarely reported (but see Roberts et al., 2012).

Increasingly, life histories of fishes are being interpreted with the aid of their otoliths (ear-stones). Otoliths are small, aragonitic structures that form part of the hearing and balance system in teleost fishes. Calcium carbonate is precipitated daily on a protein matrix, mediated by enzyme reactions (Söllner et al., 2003). Because otoliths grow incrementally, following circadian rhythms (Mugiya et al., 1981; Pannella, 1971), daily and annual growth bands are possible to resolve microscopically, a fact that revolutionized fish age and growth analysis ca. 40 years ago. With the availability of high precision, fine-scale analytical instrumentation, otolith chemistry analysis has grown over the past 15–20 years into a major research area. Indeed, well over half of the papers presented at the 4th International Otolith Symposium in 2009 concerned otolith chemistry. The combination of aging structures and chemical analyses provides a powerful set of forensic tools to examine fish life histories (Campana and Thorrold, 2001; Elsdon et al., 2008).

Most of the applications of otolith chemical analysis concerns either (1) stock discrimination (e.g., Campana, 2005) or (2) movement and provenance (e.g., Brazner et al., 2004; Walther and Limburg, 2012).

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However, other applications are possible. In particular, otolith chemistry appears to bear promise of retrospective tracking of whether and how fish interact with hypoxic/anoxic “dead zones” through analysis of the trace element manganese, typically quantified in ratio to calcium, the major divalent cation in otoliths. Most trace elements are taken up into otoliths in dissolved form, via passage over the gills or through the gut wall, through the bloodstream, and into the endolymph where they are precipitated into the otolith (Campana, 1999). The dissolved forms of manganese are the reduced species  $\text{Mn}^{2+}$ , which is thermodynamically stable in anoxic and low pH waters, and  $\text{Mn}^{3+}$ , which is an intermediate in the microbially mediated production of Mn oxides (Tebo et al., 2004; Trouwborst et al., 2006). Unlike iron, which has comparatively faster oxidation kinetics, dissolved Mn may remain in solution for periods of days at a time, even in the presence of oxygen (Pakhomova et al., 2007; Thamdrup, 2000). Thus, if a fish is present under amenable redox conditions, it may incorporate dissolved Mn into its otoliths or other biomineralized structures such as bone. This has been noted in the literature (Dorval et al., 2007; Fodrie and Herzka, 2008; Mohan et al., 2012; Moreau et al., 1983; Thorrold and Shuttleworth, 2000). Further, Limburg et al. (2011) found a significant relationship between otolith Mn:Ca in cod caught in the Baltic Sea and the intensity of hypoxia (as indexed by the area of hypoxic/anoxic bottom, Savchuk, 2010).

### 1.1. Manganese as a proxy for hypoxia

Although the biogeochemistry of manganese is largely well understood (see Reddy and DeLaune, 2008 for an excellent review; Fig. 1), the mechanism of manganese uptake in otoliths is still regarded as somewhat controversial. For one thing, manganese can be found in high concentrations in the primordia (nucleation points in the core) of otoliths of some fish species (e.g., Ben-Tzvi et al., 2007; Brophy et al., 2004; Ruttenberg et al., 2005), sometimes along with other trace elements (Limburg et al., 2013). The mechanism of incorporation appears to be maternal transfer of manganese to the embryo, and subsequent uptake into the primordia that nucleate in the otolith core. However, this does not preclude other, external influences later in the fish's life, such as encountering water with elevated dissolved manganese. To

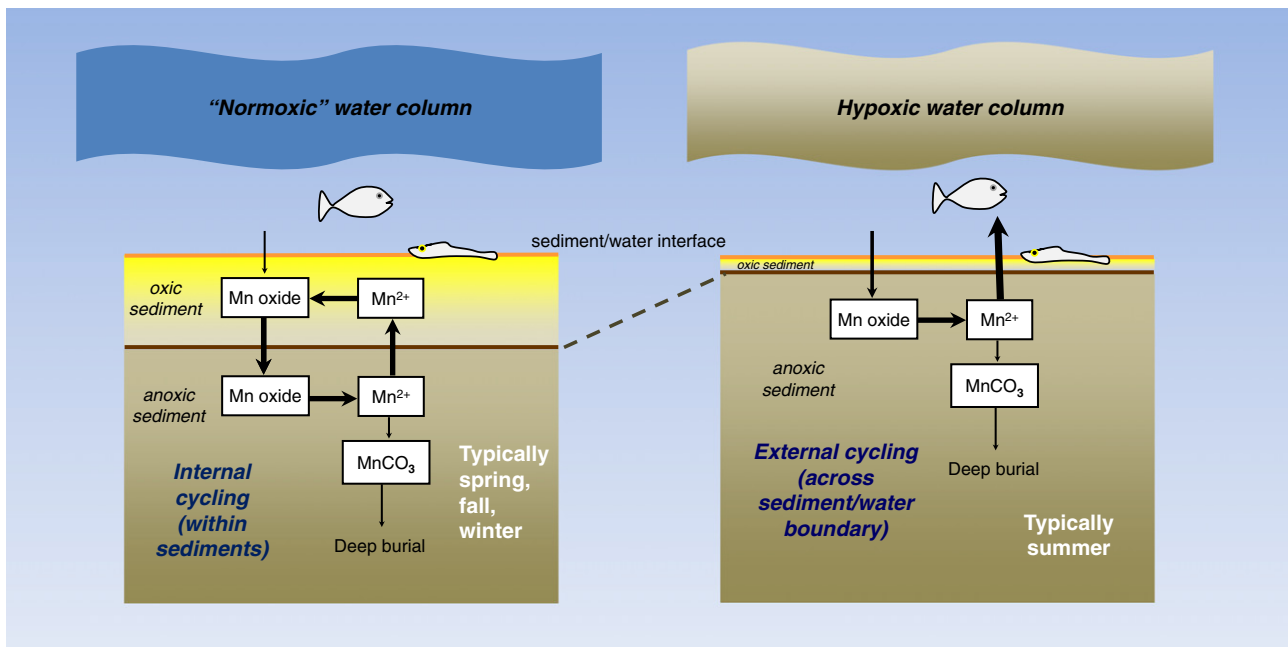
date, most support for the ability of otoliths to incorporate manganese in proportion to dissolved concentrations has come from comparisons of wild fish, with few experimental validations (Dorval et al., 2007; Forrester, 2005; Limburg et al., 2011; Mohan et al., 2012).

### 1.2. Alternate proxies of hypoxia

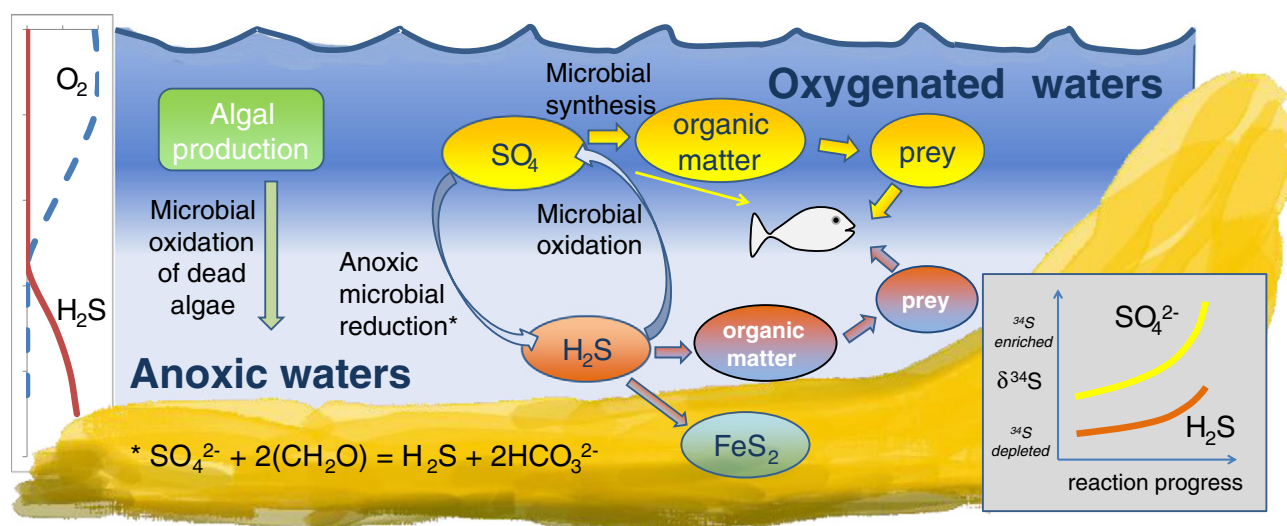
Besides manganese, a number of candidate chemical constituents may be useful to provide insights into the redox state of environments that fish live in. Of these, inorganic iodine presents as iodide ( $\text{I}^-$ , reduced form) and iodate ( $\text{IO}_3^-$ , oxidized form) in seawater, meaning iodate should dominate in oxygenated waters with a shift to iodide in hypoxic waters. Modern oxygen minimum zones are well-known for iodine speciation changes (Rue et al., 1997), and both I and Mn are among the elements most sensitive to deoxygenation. A recent study (Lu et al., 2010) reported that iodate, not iodide, is incorporated in synthesized calcite and marine biogenic carbonate. I/Ca ratios of marine carbonates decrease during periods of hypoxia; and therefore there is potential for otolith I/Ca to serve as another carbonate-based redox proxy. Iodine can be found in different types of marine biogenic carbonates such as corals and foraminifera shells (Lu et al., 2010); thus, it could be expected to be present in otoliths, although it is rarely studied.

Reduced iron ( $\text{Fe}^{2+}$ ) could conceivably also be incorporated into otoliths. Iron is similar to manganese in redox sensitivity, but once  $\text{Fe}^{2+}$  is exposed to oxygen, it readily forms particulate  $\text{Fe}^{3+}$  (Reddy and DeLaune, 2008) and thus is no longer available in dissolved form. In our experience as well as from the literature, iron is rarely detected in otoliths, and when it has been reported (e.g., Gauldie et al., 1980) it is not considered in relation to hypoxia.

Finally, constituents of the proteinaceous component of otoliths could be redox-sensitive. In particular, sulfur isotopic composition could be a marker for suboxic environments because sulfide is produced in anoxic environments (Fig. 2) and sulfide is depleted in the heavier  $^{34}\text{S}$  isotope due to fractionation during sulfate reduction to sulfide (Canfield, 2001; Chambers and Trudinger, 1979). Unlike most trace metals, S incorporation into otoliths is hypothesized to be taken up primarily from diet, rather than directly from water (Weber et al., 2002). We hypothesize that this depleted sulfur isotopic composition could



**Fig. 1.** Manganese cycling in sediments and water in normoxic (left) vs. hypoxic (right) conditions. In the former, oxic surficial sediments act as a cap, promoting internal cycling: Mn cycles between oxic and anoxic forms within the sediments. In hypoxic/anoxic conditions, little or no oxic sediment exists; dissolved Mn can therefore diffuse up into the water column. Redrawn from Slomp et al. (1997). Note that Fe cycling is similar, but Fe changes oxidation state more rapidly in the presence of oxygen (Reddy and DeLaune, 2008).



**Fig. 2.** Simplified aquatic sulfur cycle and potential source of otolith  $\delta^{34}\text{S}$  variation under oxic and anoxic conditions. The decomposition of algae and other organic matter can create anoxic conditions, under which sulfate is reduced by bacteria. The resulting hydrogen sulfide is depleted in  $^{34}\text{S}$  relative to the sulfate (see inset graph), resulting in a potential source of low  $\delta^{34}\text{S}$  organic matter, which can be passed through the food web and end up in the otoliths of fish in anoxic waters. Inset diagram (after Fry, 2006) shows changes in sulfur stable isotopic ratios in  $\text{SO}_4^{2-}$  substrate and  $\text{H}_2\text{S}$  product during sulfate reduction reaction. Small yellow arrow represents unknown but seemingly low level of otolith S derived directly from water sulfate (Mugiya and Iketsu, 1987).

be reflected in the otolith as a result of sulfide being incorporated into fish diet, or potentially a more direct but unknown route. Sulfur  $\delta^{34}\text{S}$  has been quantified in otoliths and used to distinguish hatchery from wild fish (Johnson et al., 2012; Weber et al., 2002).  $\delta^{34}\text{S}$  was used to identify different dietary sources, and thereby, provenance. Redox sensitivity per se was not explored in those studies.

### 1.3. Aim

In this paper, we provide supporting lines of evidence that post-primordium manganese found in otoliths is associated with biogeochemically favorable redox conditions, and in particular, can be used as a tracer of encountering hypoxic waters. We do this by contrasting otolith Mn uptake in fishes collected in contrasting hypoxic and normoxic waters. Further, we demonstrate the association in otoliths of other chemical constituents that are also known to be affected by hypoxia. Finally, we propose a means of quantifying hypoxia exposure by a set of readily measured characteristics of manganese uptake in otoliths.

## 2. Material and methods

### 2.1. Species used and sampling sites

In this paper we document a number of fish species, ranging across families and from freshwater to marine environments, whose otoliths were analyzed for evidence of potential hypoxia markers. Although one of us (KL) has explored the phenomenon in a wider array of taxa, our examples here include Atlantic cod *G. morhua* and European flounder *Platichthys flesus*, both from the brackish Baltic Sea. The Baltic Sea, although historically subject to periodic hypoxia and anoxia episodes typically in deeper areas (Zillén et al., 2008), has increased incidence of seasonal hypoxia since the 1960s (Conley et al., 2009; Savchuk, 2010). Cod, a demersal species, and benthic/demersal flounder use both shallow (generally less hypoxic, although see Conley et al., 2011) and deeper (generally more hypoxic) zones. Cod collections are reported in Limburg et al. (2011); European flounder were collected in February and March of 2010 and 2011 to monitor deep (>60 m) and shallow (<10 m) water spawners (Nissling and Dahlman, 2010). Yellow perch *Perca flavescens* is a widespread freshwater species in North America. For this study, specimens from Lake Erie (one of the Laurentian Great

Lakes in North America) were collected in the lake's western and central basins in October 2012; the central basin is seasonally stratified and hypoxic, whereas the western basin is periodically ventilated by wind mixing (Rucinski et al., 2010; Zhou et al., 2013). The amphidromous Atlantic croaker *Micropogonias undulatus* were collected in the northern Gulf of Mexico in fall 2010. During the 2010 cruise, a station east of the Mississippi River delta was severely hypoxic, while another station west of the delta was normoxic. Yellow perch and Atlantic croaker were all Age-1 +; Baltic flounder ranged in age from Age-4 to Age-9, but only that portion of the otolith corresponding to the first two years of life (i.e., through Age-1) were included in this analysis.

Additionally, we use two individuals to make points about (a) growth rate and (b) the uptake of redox-sensitive metals, respectively. For growth rate effect we selected an individual anadromous alewife *Alosa pseudoharengus*, collected in a coastal New England pond which experiences seasonal hypoxia. Finally, we present an unusual otolith from a winter flounder *Pseudopleuronectes americanus* collected in Little Neck Bay, Long Island, NY. Alewives are anadromous herrings, and winter flounder are estuarine/marine flatfish.

### 2.2. Otolith preparation and chemical analyses

In all species, the largest pair of otoliths (sagittae) were removed, cleaned, sectioned to expose the cores, and finely polished to at least 3  $\mu\text{m}$ . Sample sizes were between 6 and 10 fish per species collected from either hypoxic or normoxic sites (Table 1).

Otolith chemistry was quantified by several means. Some data were collected by scanning X-ray fluorescence microscopy (SXFM) at the Cornell High Energy Synchrotron Source in Ithaca, NY. This method uses high-energy X-rays generated by a synchrotron, as described in Limburg et al. (2007, 2011). Briefly, a 16.1 keV X-ray beam is produced with a multilayer monochromator (0.6% bandpass) with a photon flux of approximately  $10^{11}$  counts  $\text{s}^{-1}$ , and focused with a single-bounce, glass monocapillary to produce a uniform beam in a spot that ranged from 20 to 100  $\mu\text{m}$ , depending on the size of the otolith specimen and degree of resolution desired. Fluorescent X-rays were collected for a fixed amount of time (typically 1–10 s) with an energy-dispersive Vortex silicon drift detector fitted with an aluminum foil attenuator to reduce the calcium counts and increase sensitivity to trace elements. Calibrations were made using an in-house standard made of finely powdered otoliths pressed into a pellet (Limburg et al., 2011). Data



**Table 1**

A. Mean Mn:Ca ratios and 95% confidence intervals for Lake Erie yellow perch *Perca flavescens*, Baltic flounder *Platichthys flesus*, and Gulf of Mexico Atlantic croaker *Micropogonias undulatus*. B. ANOVA results for Mn:Ca as a function of fish species and status (= hypoxic or normoxic). Overall  $R^2 = 0.59$ . SS = sum of squares, df = degrees of freedom, MS = mean squared.

A					
Species	Status	Mean Mn:Ca	95% c.i.	N	
Yellow perch	Normoxic	0.0099	0.0124	6	
Yellow perch	Hypoxic	0.0183	0.0124	6	
Baltic flounder	Normoxic	0.0228	0.0096	10	
Baltic flounder	Hypoxic	0.0531	0.0096	10	
Atlantic croaker	Normoxic	0.0038	0.0096	10	
Atlantic croaker	Hypoxic	0.0131	0.0101	9	
B					
Effect	SS	df	MS	F	p
Intercept	0.0197	1	0.020	86.407	0.000
Species	0.0093	2	0.005	20.372	0.000
Status	0.0031	1	0.003	13.615	0.001
Species * status	0.0014	2	0.001	3.039	0.058
Error	0.0103	45	0.000		

reduction and processing were completed using PyMCA (Solé et al., 2007) and in-house software developed at CHESS to produce the maps and spatially explicit numerical output (mass fraction). Two-dimensional maps of each element were produced by rastering across the sectioned otolith (e.g., Fig. 4). The data were saved as matrices; trace element-to-calcium ratios were calculated by dividing a trace element map (matrix) by the corresponding calcium map. Data transects may be extracted by importing the maps into ArcMap 10.1 (ESRI, 2012) as raster files, and using the “Stack Profile” tool in the 3D Analyst Tools routines.

Data were also collected by means of laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS). Yellow perch, Baltic flounder, and alewife otoliths were analyzed at the College of Environmental Science and Forestry in Syracuse, NY. A New Wave (ESI) 193-nm solid state laser was used to ablate material in a transect that either began at the otolith core (birth) and extended to the outer edge (death), or extended from edge to edge, crossing the core. The ablated material was transported in a He–Ar carrier gas and analyzed with a PerkinElmer Elan DRC-e quadrupole ICPMS. For these analyses, otoliths were typically analyzed in transverse sections, using spot sizes appropriate to the size of the otolith (e.g., 35  $\mu\text{m}$  for small otoliths and 75  $\mu\text{m}$  for large ones), 3  $\mu\text{m s}^{-1}$  travel time, 10 Hz, and 70% power. The same in-house standard, as well as a standard developed by the U.S. Geological Survey, MACS-3 (USGS, 2013), were used for calibrations and instrument drift corrections. Several otoliths were analyzed on both SXFM and LA-ICPMS platforms.

Atlantic croaker otoliths were analyzed at the Jackson School of Geosciences at the University of Texas at Austin. An Agilent 7500ce quadrupole based ICP-MS (ICP-Q-MS) coupled to a New Wave UP 193-FX laser with a 25- $\mu\text{m}$  laser spot diameter and 5- $\mu\text{m s}^{-1}$  scan speed was used to quantify  $^{43}\text{Ca}$  and  $^{55}\text{Mn}$  from transects ablated across otoliths following an initial pre-ablation ablation (35- $\mu\text{m}$  spot diameter, 10% power, 25- $\mu\text{m s}^{-1}$  scan speed) to remove surface contamination. Laser transects were initiated at the otolith core and run parallel to the sulcus to the otolith edge. MACS-3 calcium carbonate and NIST 612 glass certified standards were run at the beginning and after every 60 min of analysis to convert raw intensity counts to concentrations (using MACS-3), and correct for instrument drift and assess analytical precision (with NIST 612). Elemental intensity count data was converted to concentrations using the Trace Elements IS Data Reduction Scheme in the software Iolite that uses calcium (as 37.69 wt.% in aragonite) as an internal standard (Paton et al., 2011) and then converted to molar ratios. Relative Standard Deviation (RSD) based on repeated measurements of the NIST 612 standards was Mn:Ca = 12.3%.

Sulfur isotopic analysis was performed on a select subset of otoliths of *G. morhua*, *M. undulatus*, and *P. flesus* using a modified secondary ion mass spectrometry (SIMS) method of Weber et al. (2002) at the UCLA W.M. Keck Foundation Center for Isotope Geochemistry featuring a CAMECA ims1270. A  $\text{Cs}^+$  primary ion current of  $\sim 7$  nA was focused to an  $\sim 30$   $\mu\text{m}$  analysis spot on the sample with an normal-incidence electron gun tuned for self-compensating charge neutralization in the analysis crater. The secondary ions  $^{32}\text{S}^-$  and  $^{34}\text{S}^-$  were collected in a static collection mode using two Hamamatsu miniature electron multipliers. Approximately  $10^4$  counts per second of  $^{34}\text{S}^-$  counts were obtained on the unknowns, and counts were integrated for 200 s. Isotopic ratios were calculated after a dead-time correction (65 ns). SIMS instrumental mass fractionation (IMF) for  $^{34}\text{S}/^{32}\text{S}$  using two EM detectors was corrected for each otolith individually using  $\delta^{34}\text{S}$  composition of +18‰ for the average of spots 1–3 and 10–12 for *M. undulatus* and all points ( $n = 16$ ) for *P. flesus*.

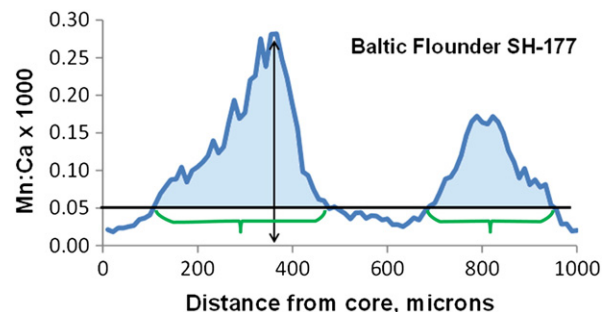
$$\text{IMF} = \left[ \frac{\left( \frac{^{34}\text{S}}{^{32}\text{S}} \right)_{\text{raw}}}{\left( \frac{^{34}\text{S}}{^{32}\text{S}} \right)_{\text{cor}}} \right] \quad (1)$$

For both otoliths, IMF was very similar, and averaged 0.953. We note that the relative magnitude in  $\delta^{34}\text{S}$  variation for each otolith is independent of the IMF correction. Corrected ratios,  $(^{34}\text{S}/^{32}\text{S})_{\text{cor}}$  were converted to  $\delta^{34}\text{S}$  values in parts per mil (‰) relative to Canyon Diablo Troilite (CDT);  $(^{34}\text{S}/^{32}\text{S})_{\text{CDT}} = 0.044163$  (Ding et al., 2001) using:

$$\delta^{34}\text{S}_{\text{cor}} = \left[ \frac{\left( \frac{^{34}\text{S}}{^{32}\text{S}} \right)_{\text{cor}}}{\left( \frac{^{34}\text{S}}{^{32}\text{S}} \right)_{\text{CDT}}} - 1 \right] * 1000\text{‰} \quad (2)$$

### 2.3. Statistical analyses

Because of the highly variable and probabilistic nature of encountering hypoxia (and thus elevated ratios of Mn:Ca should be found in otoliths), we developed two sets of metrics to assess whether expectations of higher Mn:Ca prevail in fishes collected in known hypoxic areas vs less hypoxic/normoxic areas. First, we defined a set of metrics to characterize the magnitude, frequency, and duration of putative exposure. An otolith transect of Mn:Ca ratios can be characterized by (a) a maximum Mn:Ca value; (b) the number of continuous occurrences of exceeding a threshold Mn:Ca value; and (c) the “duration,” or fraction of the transect that is occupied by the exceedences. These are illustrated in Fig. 3. Metrics were developed for Baltic flounder, Atlantic croaker, and yellow perch. Exceedence thresholds were set by visual inspection of transects in each species; for Baltic flounder the threshold was set to 0.05; for Atlantic croaker, 0.04; and for yellow perch, 0.03 Mn:Ca ratio. Twenty



**Fig. 3.** Illustration of quantification of descriptive metrics of exposure to hypoxia as indicated by the otolith proxy of Mn:Ca ratios. Double-headed arrow indicates “maximum magnitude” metric; the two shaded areas indicate events above a defined threshold (here defined as exceeding  $\text{Mn:Ca} > 0.05 \times 10^{-3}$ ). The summed distance of the two brackets, divided by the total distance, provides a third metric “duration of events”.

flounder, 19 croaker, and 12 perch were used to quantify these metrics and for the following analyses.

We tested whether these three characteristics of Mn:Ca transects were greater in fish captured in hypoxic waters with factorial multivariate analysis of variance (MANOVA) where these three metrics served as the dependent variables and species and status (hypoxia or normoxia) were the independent variables. The data were not transformed as the residuals were approximately normally distributed. In addition, univariate ANOVA was performed, testing for whether overall mean Mn:Ca values differed as a function of species or status. These tests were performed with Statistica Version 12 (StatSoft, Inc., 2013).

We also conducted an analysis similar to a “flow duration curve” analysis in hydrology (Black, 1996). The method is used to characterize the frequency of occurrence of different flow events in a hydrologic time series, but can be adapted to characterize other data in time series as well, including the life history transects of trace elemental chemistry in otoliths. The “concentration exceedence” curves are analogous to cumulative distribution functions. Concentration exceedence curves were computed for Baltic flounder, Atlantic croaker, and yellow perch otolith Mn:Ca transects with a flow duration curve function in the R programming language (R Development Team, 2012) available from a hydrology tutorial (Rwiki, 2009).

All Mn:Ca transect data are included as online Supplementary Data S1.

### 3. Results

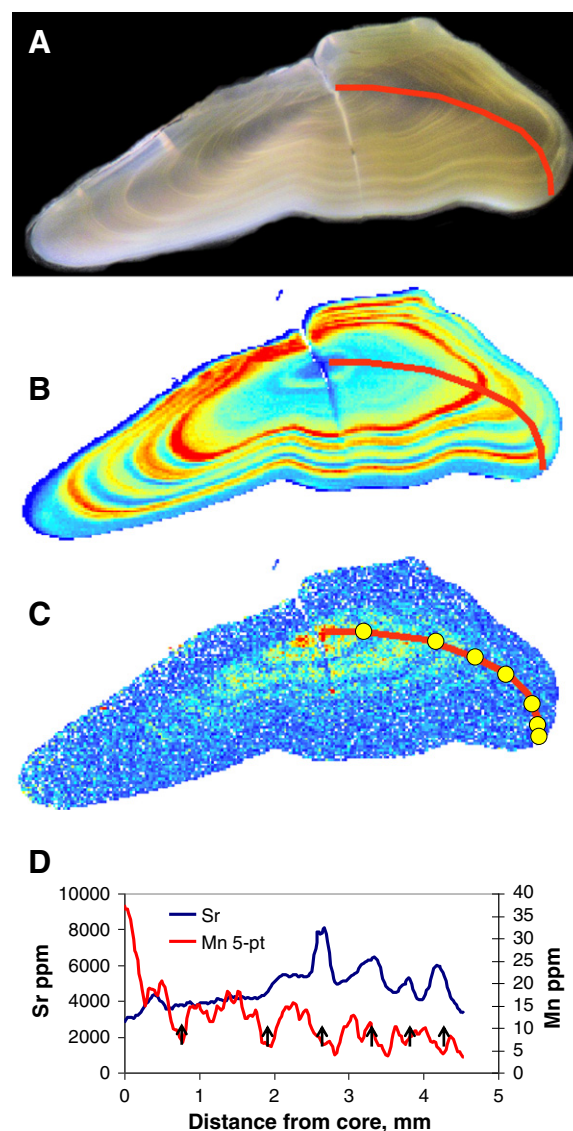
#### 3.1. Manganese as a proxy

Whereas evidence that otolith manganese can serve as a proxy for hypoxia exposure has been presented elsewhere (Limburg et al., 2011), here we present some more data to suggest constraints on interpretation, but also to suggest the general utility of the proxy.

##### 3.1.1. Evidence for growth effects on Mn uptake

Limburg et al. (2011, online Supporting Information) presented data suggesting that manganese uptake in otoliths is to some extent affected by growth rate, and developed a simple mathematical model to describe uptake as a function of both fish growth rate and environmental availability of manganese. Although we will not elaborate further on the model here, we show how growth rate is correlated with declines in otolith manganese. In adult cod *G. morhua* from the Baltic Sea caught in the early 2000s (a period of intense summertime hypoxia), accretion of otolith Mn often shows a repeated pattern of increase in the summer/fall and decline in winter otolith formation; yet a gradual decrease in concentrations is seen over the course of years as the fish ages (Fig. 4). Limburg et al. (2011) noted that the decline could also be due in part to an averaging effect, because the analytical beam has a constant diameter but traverses increasingly smaller growth bands in the outer parts of otoliths (Fig. 4A).

In a second example (Fig. 5), daily growth rate of an otolith of a juvenile alewife (*A. pseudoharengus*) was found to correlate approximately with changes in Mn:Ca ratios. This fish came from a coastal pond on Cape Cod, Massachusetts, where water quality was poor and hypoxic episodes have been recorded, although not concurrently with the study for which this fish was collected. Of note is the visible change in opacity, seen in the optical image as a translucent zone about midway through the otolith. All fish collected from this pond showed the same translucent zone. Microscopic examination revealed very thin daily growth increments in this zone, corresponding to slow growth likely brought on by stress. A corresponding decline in Mn:Ca ratios was observed, and was not due to averaging by the laser beam. Although we do not have corresponding oxygen data, the pond is known to undergo summertime hypoxia and thus we postulate that the stress was brought about by elevated water temperatures and low



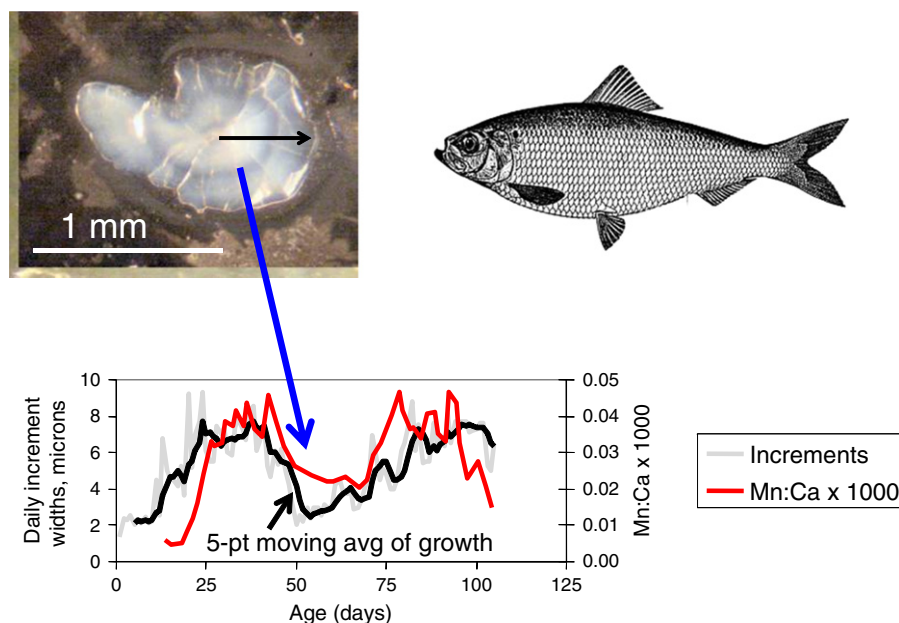
**Fig. 4.** Otolith of a Baltic cod, Age 7, total length 99 cm, caught October 20, 2005, analyzed by SXFM. A. Optical image (transmitted light). B. Strontium map. C. Manganese map; yellow dots mark winter banding (annulus formation). D. Sr and Mn (5-point smoothed) concentration data extracted from the maps along a transect illustrated as a red line on the optical image and trace elemental maps; arrows mark locations of annuli. Note the general decline in Mn with age, whereas Sr increases with age at first and then varies as the fish migrates.

dissolved oxygen. The point, however, is that growth rate appears to mediate manganese incorporation into the otolith matrix.

##### 3.1.2. Proxy comparison among species

Manganese uptake by otoliths not only depends on environmental concentrations, but also is to some extent affected by growth (Figs. 4 and 5). Given that fish growth decelerates with age, we have found that the portions of otoliths that provide the highest resolution of manganese dynamics are generally the first and second annual growth zones (i.e., from birth to the second annulus formation). Thus, for comparisons among systems, it may be useful to sample young fish, or to sample the portions of otoliths corresponding to the first two years of life, i.e., ages 0 and 1.

Accordingly, we now demonstrate that otolith manganese (in ratio to calcium) appears to be a good proxy for hypoxic conditions across taxa and ecosystems ranging from fresh to marine waters. For this, we used the Lake Erie yellow perch, Baltic flounder, and northern Gulf of



**Fig. 5.** Otolith of a young-of-the-year juvenile alewife collected in a polluted coastal pond in Cape Cod, Massachusetts. Black arrow on micrograph denotes transect used to measure daily growth increments and trace elemental chemistry, shown in the bottom panel. Blue arrow points from translucent zone on the micrograph to the corresponding decrease in daily increment widths (growth rate) and Mn:Ca.

Mexico Atlantic croaker specimens collected in contrasting hypoxic and normoxic areas (see Section 2.1).

Despite great individual variability, otoliths of fish collected in hypoxic waters tend to display not only greater magnitudes of Mn:Ca than those of fish collected in oxygenated water (Fig. 6), but also greater variability, frequency, and duration of exposure. These features appear to characterize the nature of hypoxia exposure in fish that can move about, but are still likely to encounter hypoxic water. In this comparison, which controls for a growth effect by restricting the analyses to younger ages when all fish grow relatively fast, Mn:Ca was higher overall in hypoxic systems, but a species effect is apparent as well ( $p \leq 10^{-3}$ ; ANOVA, Table 1). In addition, the three metrics of magnitude, frequency, and duration of elevated Mn:Ca were significantly higher ( $p < 10^{-3}$ ) in fishes caught in hypoxic areas than in normoxic ones (MANOVA, Table 2),

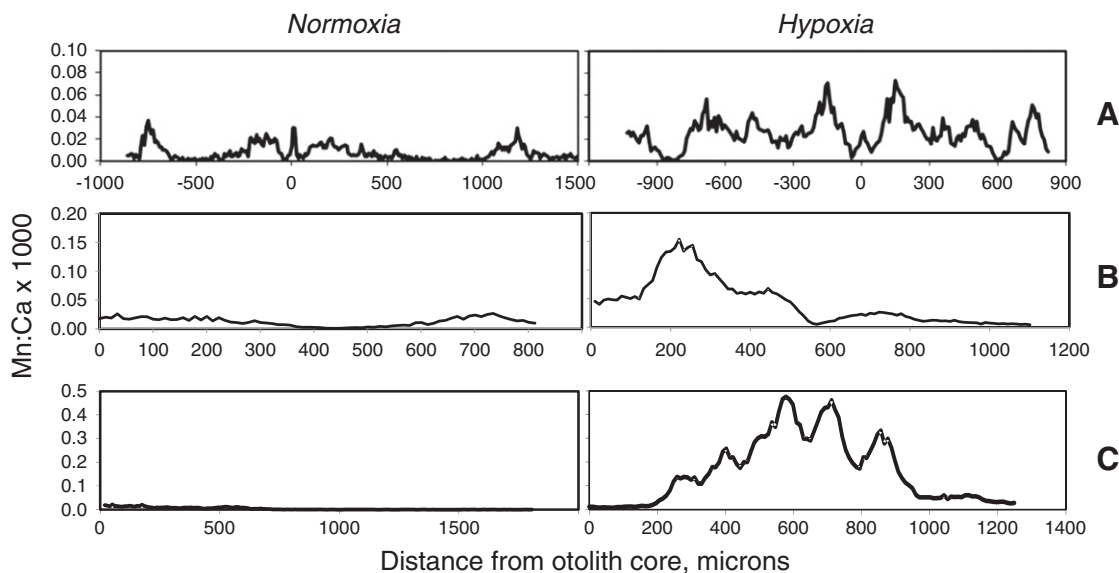
and the interaction between species and hypoxia status was significant as well ( $p < 10^{-3}$ ).

We also used “concentration exceedance curves,” adapted from hydrological flow frequency analysis, to characterize the cumulative frequency of occurrences of Mn:Ca in these fishes (see Materials and methods). When all the data for each group are analyzed in this manner, the percent of the data exceeding any given Mn:Ca ratio is always greater in the hypoxic group (Fig. 7).

### 3.2. Other chemical proxies for hypoxia

#### 3.2.1. Spatial analysis of a deformed winter flounder otolith

In a study of juvenile winter flounder *P. americanus* use of embayments around Long Island, New York (USA), one of us (GJ) discovered



**Fig. 6.** Mn:Ca transects across representative transverse sections of (A) Lake Erie yellow perch, (B) Baltic flounder, and (C) Gulf of Mexico Atlantic croaker. The left panels are otoliths of fish caught in normoxic water, while the right panels are from fish caught in hypoxic water. Note different vertical axis scales among compared pairs. All transect data may be found in online Supplemental Data S1.

**Table 2**

A. MANOVA results for maximum Mn:Ca ratios (maximum magnitude), number of events exceeding a given Mn:Ca threshold (frequency), and duration of events computed as microns of Mn:Ca exceeding the threshold/total transect length for otoliths of Lake Erie *Perca flavescens*, Baltic flounder *Platichthys flesus*, and Gulf of Mexico Atlantic croaker *Micropogonias undulatus*. See Fig. 3 for definitions of metrics. Threshold Mn:Ca values were 0.03 for yellow perch, 0.04 for Atlantic croaker, and 0.05 for Baltic flounder. B. Multivariate tests of significance (MANOVA).

A									
Effect	Level of factor	Level of factor	N	Max. Mn:Ca	95% c.i.	Number of events	95% c.i.	Duration of events	95% c.i.
Total			51	0.093	0.027	1.51	0.479	12.8%	0.049
Species	Yellow perch		12	0.057	0.014	2.92	1.164	10.8%	0.062
Species	Baltic flounder		20	0.134	0.051	1.25	0.566	22.4%	0.104
Species	Atlantic croaker		19	0.072	0.049	0.89	0.785	4.0%	0.040
Status	Hypoxic		25	0.137	0.046	2.40	0.781	21.6%	0.081
Status	Normoxic		26	0.051	0.023	0.65	0.360	4.4%	0.036
Species * status	Yellow perch	Hypoxic	6	0.073	0.020	4.17	1.808	16.3%	0.105
Species * status	Yellow perch	Normoxic	6	0.042	0.009	1.67	0.857	5.3%	0.064
Species * status	Baltic flounder	Hypoxic	10	0.188	0.077	1.90	0.856	36.6%	0.150
Species * status	Baltic flounder	Normoxic	10	0.079	0.059	0.60	0.603	8.2%	0.092
Species * status	Atlantic croaker	Hypoxic	9	0.123	0.102	1.78	1.574	8.4%	0.081
Species * status	Atlantic croaker	Normoxic	10	0.027	0.011	0.10	0.226	0.1%	0.002

B						
Effect	Test	Value	F	Effect df	Error df	p
Intercept	Wilk	0.32655	29.56	3	43	0.00000
Species	Wilk	0.25313	14.16	6	86	0.00000
Status	Wilk	0.63020	8.41	3	43	0.00016
Species * status	Wilk	0.57469	4.57	6	86	0.00045

an unusual, deformed otolith (Fig. 8A). The otolith was found on the blind side of the fish skull and was notably smaller than the normal otolith on the eyed side. The otolith had a number of blue-colored inclusions, and was shaded pale blue on its exterior. Of thousands of otoliths examined overall, and hundreds of winter flounder otoliths in

particular, we have only encountered this solitary otolith deformed in this way.

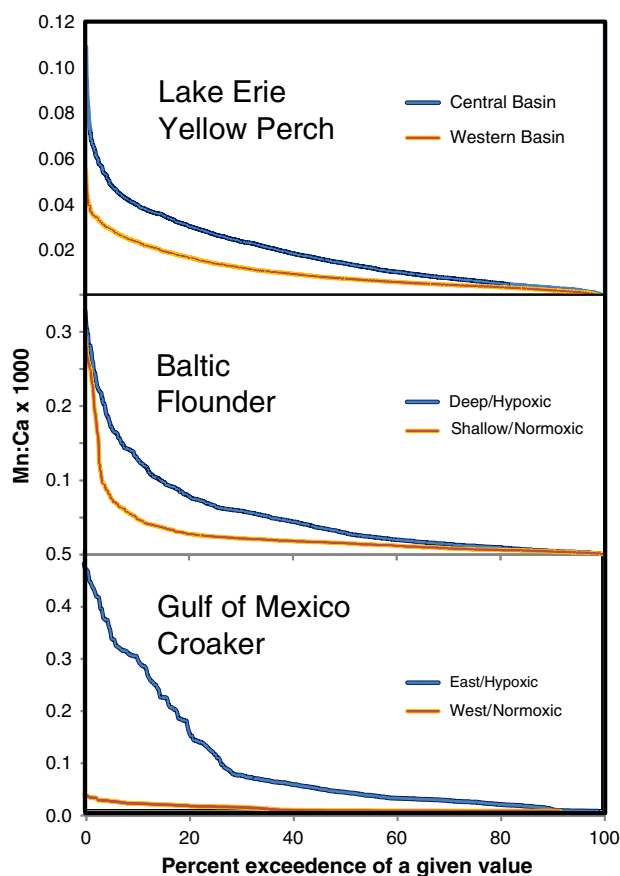
SXFM analysis of the sectioned otolith revealed that the inclusions contained elevated concentrations of iron, manganese, zinc, and bromine (Fig. 8A and B), but not copper, as the color suggested (Fig. 8B). The levels of Sr:Ca were similar to the other sagittal otolith, indicating that the otolith itself was composed of aragonite and not vaterite.

### 3.2.2. Iodine as a potential proxy

We have conducted a number of analyses that have included iodine as a potential proxy indicator of periods of hypoxic conditions during fish life cycles. Our results to date have been somewhat mixed, in part due to the analytical limitations of the low resolution quadrupole mass spectrometer available to us (note that it is not possible to detect iodine with SXFM in this material). Our most promising results have been in fish from brackish water (Fig. 9), as fish in fresh water do not accumulate I and fish analyzed to date from full-strength seawater did not display variation in I:Ca. Iodine concentrations are about an order of magnitude lower than manganese concentrations. Regardless of the fluctuations, the smoothed I:Ca declines when Mn:Ca increases and vice-versa.

### 3.2.3. Sulfur stable isotopes

Analysis of several cod otoliths that had previously been used in SXFM analyses had suffered severe sulfur loss, perhaps due to X-ray irradiation or subsequent heating for sample mounting. However, two otoliths (one from a Gulf of Mexico Atlantic croaker *M. undulatus* and one from a Baltic flounder *P. flesus*), previously analyzed only with LA-ICPMS, contained quantifiable S. The two specimens that were successfully analyzed via secondary ion mass spectrometry showed different patterns of  $\delta^{34}\text{S}$  in relation to parallel Mn:Ca transects (Fig. 10). The Atlantic croaker otolith (Fig. 10A) showed an inverse relationship between  $\delta^{34}\text{S}$  and Mn:Ca ratios. These results are consistent with our hypothesis that otolith  $\delta^{34}\text{S}$  could be a proxy for exposure to anoxic conditions. During normoxic periods, marine  $\text{SO}_4^{2-}$  ( $\delta^{34}\text{S} \approx 21\text{‰}$ ) via dietary sources ( $\delta^{34}\text{S} \approx 18\text{‰}$ ) is the primary source of S in the otolith. During suboxic/anoxic periods,  $\text{H}_2\text{S}$  (depleted in  $^{34}\text{S}$ ) may become a significant source of otolith S, presumably via dietary sources. In contrast, the  $\delta^{34}\text{S}$  in a Baltic flounder otolith remained moderately stable across the entire transect and did not decline in the otolith where the Mn:Ca increased (Fig. 10B). The difference between these isotopic responses



**Fig. 7.** Mn:Ca exceedence curves for Lake Erie yellow perch (top panel), Baltic Sea flounder (middle panel), and Gulf of Mexico Atlantic croaker (bottom panel). Note differences in vertical axis scales.



may suggest that sulfur is incorporated into otoliths from trophic S, as hypothesized by Weber et al. (2002).

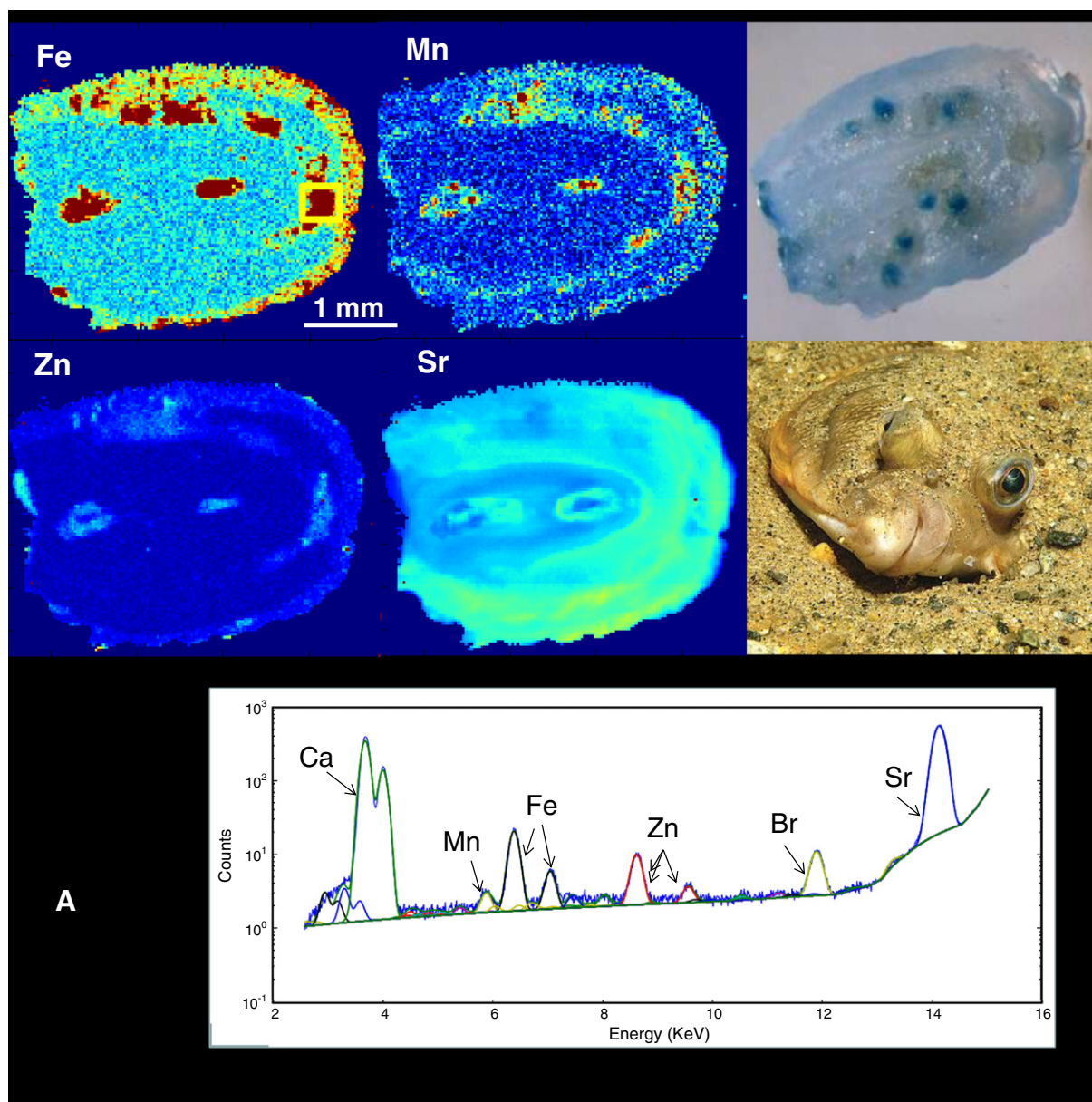
#### 4. Discussion

The results presented above provide support for the hypothesis that post-primordium otolith manganese can serve as a redox proxy. We also demonstrated that Mn:Ca appears to be affected by growth, and thus further tool development may be necessary to disentangle growth effects from the redox signature. For example, models of fish somatic growth and otolith elemental uptake show promise (Limburg et al., 2011 online Supporting Information); and bioenergetics models of otolith growth may also be of great use here (e.g., Pecquerie et al., 2012). In addition, in some taxa other trace elements clearly track growth rates and thus can be used to separate growth and hypoxia. These include salmoniform fishes, which accumulate otolith zinc proportionally to growth rate (Limburg and Elfman, 2010), and otolith magnesium

appears to track growth in silver perch *Bidyanus bidyanus* (Woodcock et al., 2012) as well as in some flounder species (Limburg, unpublished data).

We also note that Mn:Ca within an otolith can be constrained by biomineralization processes. As Hamer and Jenkins (2007) and Mohan et al. (2014) note, Mn:Ca in the slower growing sulcal groove is often considerably lower than in faster growing otolith parts. Thus, when comparing Mn:Ca uptake among different fish, care must be taken to standardize the growth axes along which data are collected.

Nevertheless, the mechanism of manganese incorporation into otolith carbonate remains elusive. The few controlled laboratory experiments that investigated relationships between water and otolith Mn:Ca ratios have been inconclusive (reviewed in Miller, 2009). In experiments using euryhaline black bream *Acanthopagrus butcheri*, Elsdon and Gillanders (2003) spiked tanks with  $MnCl_2$  to produce large variations in Mn:Ca water concentrations across treatments, although they did not observe any significant relationship between otolith and water



**Fig. 8.** Analysis of a deformed otolith from a winter flounder captured in Little Neck Bay, Long Island, New York. (A) Collage with otolith (upper right), flounder buried in sand, trace elemental maps (upper left and center), and X-ray fluorescence spectrum, on a part of the otolith indicated in the square on the iron map. (B) Trace element:calcium transects extracted from maps with ArcGIS as indicated by colored line drawn on otolith maps to the right. Note differences in vertical axis scales. Light blue lines above the transect data indicate where the analyses intersected the blue inclusions.



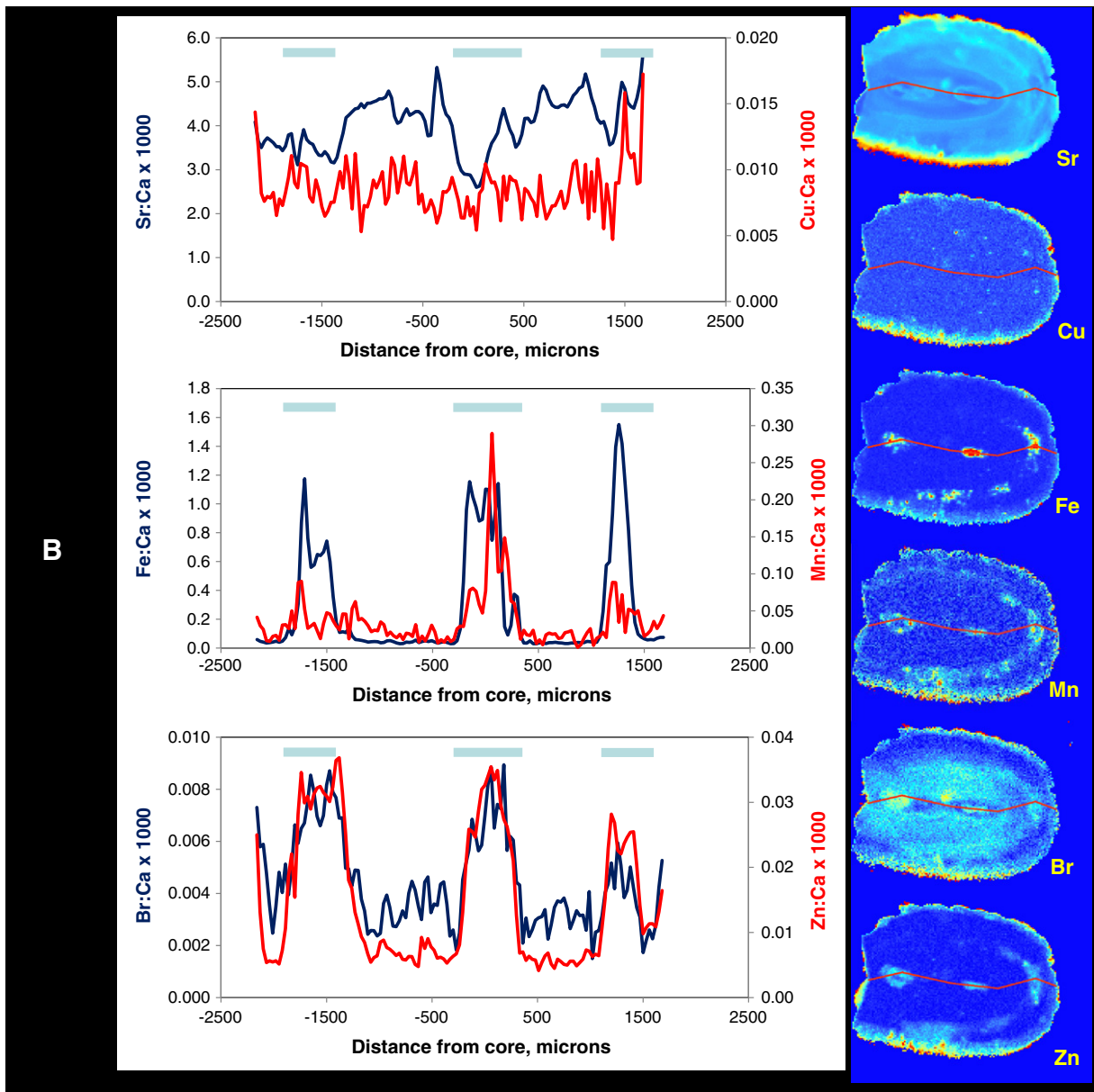


Fig. 8 (continued).

concentrations. Ratios of Mn:Ca in otoliths of the marine species black rockfish *Sebastes melanops* were similarly unresponsive in  $\text{MnCl}_2$  spiking experiments performed by Miller (2009). Collingsworth et al. (2010) spiked water with  $\text{MnCl}_2$  but observed no response in otoliths

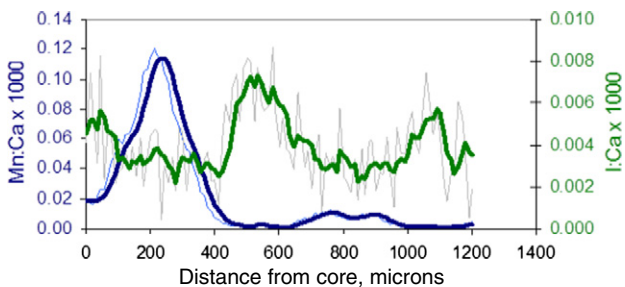
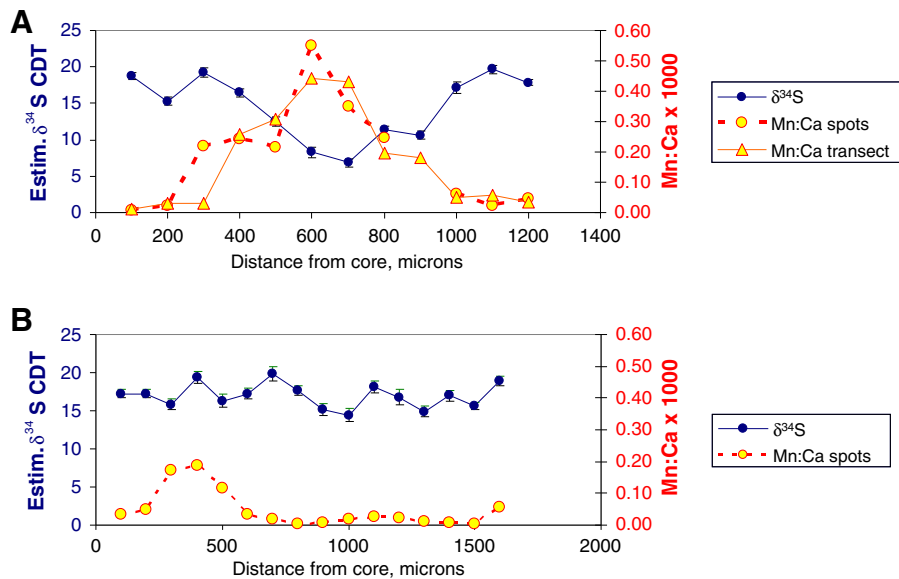


Fig. 9. Transect from an otolith of a winter flounder, showing approximately inverse relationship of Mn:Ca and I:Ca. Data were smoothed with a 5-point moving average.

of the freshwater species yellow perch *Perca flavens*, although their inability to consistently and accurately measure water Mn:Ca ratios limits the scope of the conclusions. To our knowledge, these are the only three experiments to date that have used direct manipulation of water Mn:Ca ratios via spiking, although other workers have investigated the effects of temperature and salinity manipulations on Mn uptake into otoliths with mixed results (e.g., Elsdon and Gillanders, 2002; Martin and Wuenschel, 2006). In contrast to these experiments, several field-based studies have concluded that elevated ambient Mn:Ca is associated with higher otolith Mn:Ca concentrations (Dorval et al., 2007; Forrester, 2005; Mohan et al., 2012). Other field-based studies have not detected associations between water and otolith Mn:Ca (e.g., Walther and Thorrold, 2008), although temporal decoupling between water and otolith composition due to point-based water sampling may have been a factor in these cases.

Why discrepancies exist between laboratory experiments and field studies for manganese incorporation is unknown. However, the chemical characteristics of Mn may themselves be important. As discussed



**Fig. 10.** Transects of  $\delta^{34}\text{S}$  compared to Mn:Ca in (A) an Atlantic croaker otolith from hypoxic site in the northern Gulf of Mexico and (B) a Baltic flounder otolith from a fish collected in hypoxic water. For Atlantic croaker the Mn:Ca was quantified both by point analysis as well as by extracting point estimates from a continuous line transect.

here, the speciation of Mn and production of oxides depend primarily on physicochemical parameters including oxygenation and pH. In a laboratory setting, inhibiting microbial oxidation of dissolved Mn may be difficult; this would limit the pool of bioavailable ions for uptake into the fish (Martin and Thorrold, 2005). Future experiments may need to include oxygen saturation as a manipulated variable in order to explore the importance of Mn speciation on uptake. Experimental validation of Mn uptake is made more difficult given that only a single stable isotope of Mn exists, so that isotopic spiking to resolve uptake dynamics as has been done for Sr, Ba and Mg (e.g., Woodcock et al., 2013) is difficult for Mn, although radioisotopic spiking with  $^{54}\text{Mn}$  should be possible. An additional, unexplored mechanism is the trophic transfer of Mn; this could be important particularly for fishes that consume benthic prey. Thus, the mechanism(s) of manganese incorporation into otoliths presents an unmet challenge for further experimentation.

In addition, most workers use a  $0.45\ \mu\text{m}$  filter for water chemistry analysis and operationally define the filtrate as being the dissolved fraction. However, colloids can exist in the  $0.22\text{--}0.45\ \mu\text{m}$  range and bind as much as 60% of putatively dissolved Mn in some systems (Pokrovsky et al., 2010). Researchers in either experimental or field settings may thus overestimate the true concentration of bioavailable dissolved Mn ions in some situations, which may help explain conflicting results.

Experimental confirmation (or lack thereof) notwithstanding, careful selection of natural contrasts can provide insights into the mechanisms of Mn uptake and incorporation into otoliths. Specifically, here we have documented that fishes collected in contrasting hypoxic/normoxic environments meet the expectation that Mn:Ca is elevated in fish otoliths collected in hypoxia. However, there is variation in observed Mn:Ca; some fish captured in hypoxic waters have low otolith Mn:Ca, whereas some fish captured in normoxic waters have histories recorded as moderately elevated Mn:Ca. This natural variability can be in part due to the fact that fish move about, as well as to the ephemeral nature of many hypoxic events. Additionally, very low dissolved oxygen ( $\text{DO} < 1\ \text{mg l}^{-1}$ ) may be required to achieve sediment redox potentials favoring vertical flux of  $\text{Mn}^{2+}$  from interstitial porewater to the overlying water column (Statham et al., 2005). DO levels  $< 1\ \text{mg l}^{-1}$  are below the tolerance level of most fishes and thus they might actively avoid waters approaching anoxic conditions that may contain high dissolved Mn.

We note that manganese solubility is also sensitive to pH (Eby, 2004), becoming more soluble at lower pH. Thus, acidic environments such as wetlands with a high degree of organic decomposition,

blackwater swamps, or certain lakes (e.g. Moreau et al., 1983) may also increase  $\text{Mn}^{2+}$  availability. For example, strongly contrasting otolith Mn:Ca has been observed in Amazonian fishes that make seasonal use of blackwater swamps, but not in fishes that live entirely in Amazonian “white water” of higher pH (Hermann et al., in preparation). Such swamps may enhance Mn availability through a combination of low dissolved oxygen and low pH. In marine systems, whether or not ocean acidification will make  $\text{Mn}^{2+}$  more available is an open question.

Other redox-sensitive trace elements (iron, iodine, zinc, and bromine) were shown to be incorporated into otoliths (Figs. 8 and 9). Again, the mechanisms of incorporation are not well understood, but our data suggest that these elements and isotopes became available in hypoxic conditions. In the case of the winter flounder, it is a benthic flat-fish species, with both eyes on its right (“seeing”) side. These fish are known to settle often within the upper layers of sediment (see photograph, Fig. 8A). Accordingly, we hypothesize that this individual spent a considerable amount of time with its blind side buried in the sediment, and that the sediment porewater was anoxic with elevated concentrations of available reduced ions. We do not know how the nodules we discovered in the “blind side” otolith could have formed, but hypothesize that one possible mechanism is diffusion of reduced ions across tissues into the sacculus, or uptake through the left gill, or perhaps through some damaged tissue on the blind side of the fish. This rare otolith does inform us that otolith material may interact in redox chemistry. In general, more investigations are certainly warranted in order better to understand the constraints on interpretation.

Currently, we do not know the chemical form of iodine incorporated in otoliths, and in the calcification environment. It is possible that otoliths contain mainly organic iodine instead of iodate, considering the protein matrix necessary for growth. If that proves to be the case, diet and physiology will have to be taken into account when interpreting otolith I:Ca records. Although there are many unknowns, hypoxia is the most likely cause of the inverse-correlation between Mn and I in otoliths, since these two elements have completely different biogeochemical behaviors (Kuepper et al., 2011), and being redox-sensitive is a conspicuous overlap.

Sulfur stable isotope ratios are consistent with severe hypoxia experienced by the Atlantic croaker (Fig. 10A) but not the Baltic Sea flounder (Fig. 10B). Because we expect diet to be the primary source of otolith S (Mugiya and Iketsu, 1987; Weber et al., 2002), we hypothesize that this result may be due to differences in the feeding habits of these fish.

Under this hypothesis, the Atlantic croaker would feed on prey items that incorporated the sulfide and thereby have lower sulfur isotopic composition than most pelagic organisms. By contrast, even if the Baltic Sea flounder was exposed to hypoxic conditions, it appears to have fed on prey items with normal marine sulfur isotopic composition. In addition, the fact that Mn:Ca and  $\delta^{34}\text{S}$  are de-coupled in the flounder otolith suggests that different mechanisms of uptake occurred. If  $\delta^{34}\text{S}$  is taken up in food, then Mn may be taken up from water. Alternatively, both might come from the same source (food or water) but be transported to otoliths by different mechanisms. Certainly this is an open question amenable to experimentation.

Currently, otolith manganese appears to be the best candidate for reconstruction of redox status of environments experienced by fishes. It is readily measured with widely available instrumentation at reasonable cost (i.e., LA-ICPMS). Combining otolith manganese concentrations with growth information from otolith microstructure can aid in understanding the timing, duration, frequency, and intensity of hypoxia encounters. We suggest that developing a set of metrics such as these for analysis of otolith transects, similar to analysis of storm events, may be useful to interpret fish interactions with dead zones. These data can be collected readily from otolith transects as demonstrated here. Caveats such as species-specific effects, growth effects, or physicochemical interactions remain. However, we see the promise for using this proxy as a tool to quantify other impacts of hypoxia that presently cannot be assessed, such as long-term or population-level consequences of frequent hypoxia exposure (particularly since older fish are the survivors of earlier life history events including early exposure to hypoxia). Additionally, fish and their otoliths may be regarded as “mobile monitors” of hypoxia, potentially revealing the breadth and extent of hypoxia beyond the capabilities of conventional technology.

## 5. Conclusions

How fish interact with hypoxic zones has been difficult to study. However, we have proposed that several trace elements (primarily Mn, but also Fe, I, and Zn) can be incorporated into otoliths under expectations of hypoxia, suboxia, or anoxia, and otolith  $\delta^{34}\text{S}$  may also reflect such conditions. Additionally, we propose a means of quantifying hypoxia exposure by a set of readily measured characteristics of manganese uptake in otoliths. The results we present are empirical observations but are consistent with biogeochemical theory. Much work remains to design proper experiments. Nevertheless, we are optimistic that these biogeochemical tracers in fish otoliths, primarily manganese, will be useful to examine lifetime exposures to hypoxia by individual fish. Although magnitudes of elemental uptake may vary among species, and will also be constrained by elemental or isotopic availability, such information can help to develop population- and even community-level understanding of hypoxia impacts on fishes. This understanding will enhance fisheries management in an era of increasing hypoxia.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmarsys.2014.02.014>.

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