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Food for Thought

Slave to the rhythm: seasonal signals in otolith microchemistry reveal age of eastern Baltic cod (*Gadus morhua*)

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Annual growth zones in cod otoliths from the eastern Baltic stock are less discrete than in other cod stocks leading to biased age reading, which recently led to a failure of age-based assessment in the eastern Baltic cod stock. In this study, we explored the applicability of minor and trace element patterns in cod otoliths for age determination. By first identifying elements of interest in a stock without ageing problems, western Baltic cod, we then tested their applicability on another stock without ageing problems, North Sea cod, and finally applied this knowledge to estimate age of eastern Baltic cod. In western Baltic cod, matching patterns with respect to occurrence of minima and maxima in both otolith opacity and element concentrations were found for Cu, Zn, and Rb, and inverse patterns with Mg and Mn. No match was found for Pb, Ba, and Sr. In the test stock, the North Sea cod, the same patterns in Cu, Zn, Rb, Mg, and Mn signals occurred. All eastern Baltic cod with low visual contrast between growth zones exhibited clearly defined synchronous cycles in Cu, Zn, Rb and Pb. Using a combined finite differencing method and structural break models approach, the statistical significance of the local profile minima were identified, based on which their age could be estimated. Despite extensive environmental differences between the three areas examined, the element concentrations of Cu, Zn, and Rb were strongly correlated in all individuals with similar correlations in all three areas, suggesting that the incorporation mechanisms are the same for these elements and independent of environmental concentrations.

Keywords: age estimation, Atlantic cod, microchemistry, otolith, seasonal patterns.

Introduction

Alternating opaque and translucent zones in fish otoliths representing seasonal variations in fish growth are the principal tool for routine age determination of many fish species. Transparent zones have traditionally been called "winter rings" or "annuli" and are counted to determine the fish's age (Campana, 2001). Prerequisites for using such zones for age determination are that the timing of their formation is synchronous in all individuals, across all age classes, and stock components and represents an annual recurring cycle. In most Northeast Atlantic ecosystems, pronounced variations in temperature in synchrony with the seasonal feeding patterns lead to distinct, annual patterns in otolith macrostructure (Høie and Folkvord, 2006). Within stocks of the same species, for example Atlantic cod (*Gadus morhua*) or European hake (*Merluccius merluccius*), annulus formation may vary from well-defined patterns in some stocks, to weakly defined patterns with low contrast between opaque and translucent zones in other stocks (Beckman and Wilson, 1995), or may exhibit very well-defined patterns that do not correspond to seasonal signals (de Pontual *et al.*, 2006). Whereas age determinations have high accuracy and precision in the former stocks, age estimates obtained for the latter may be described as a "qualified guess".

In eastern Baltic cod age reading problems have been documented for 40 years (ICES, 2006) and increasing inconsistencies recently

have led to the failure of an age-based assessment (ICES, 2014a). Analyses of daily increment patterns have highlighted two major problems arising when using the general guidelines for age reading: age readers seem to miss the first annulus, and presumed annuli are not always formed during winter (Hüssy, 2010; Hüssy *et al.*, 2010). This technique is very time-consuming and requires extensive expertise in both preparation and analytical methods—and is therefore not well suited as routine validation method.

The current assessment problems call for rigorous validation of age readings or the development of alternative methods to derive age and growth information. Today, a suite of technologies and methods is available for corroborating zone formation or direct age validation (see review in Campana, 2001). Corroboration of fish age may be achieved based on indirect methods that validate the periodicity of annulus formation and timing of the first annulus through analyses of edge type, marginal increment ratio, daily increments between annuli, length frequency progression, etc. (Campana, 2001). They are well suited to confirm annulus formation when precision among age reader interpretations is high. But as they do not validate the entire age of the fish, they are not very useful when growth zone formation does not follow the general pattern, e.g. in Baltic cod. Direct methods of corroborating fish age rely on external and internal tagging of the fish with subsequent recapture, while otolith element composition holds great potential for validating the total age of the fish. Bomb radiocarbon, based on the fallout from nuclear bomb testing, is one of the most reliable age validation techniques for long-lived species (Kalish *et al.*, 1996, 1997; Campana, 1997, 1999; Campana and Jones, 1998). Also radiochemical dating of the otolith core, based on the decay of naturally occurring radioisotopes, is primarily suited for long-lived species (Campana *et al.*, 1993; Kastelle *et al.*, 1994; Burton *et al.*, 1999; Campana, 1999; Cailliet *et al.*, 2001). Similarly, dating of the otolith core based on interannual variations in $\delta^{18}O$ is useful to validate the age of short-lived species (Upton *et al.*, 2012). Høie and Folkvord (2006) demonstrated the suitability of stable oxygen isotopes as validation technique in Atlantic cod, owing to the link between $\delta^{18}O$ incorporation and environmental temperature.

The present study presents a potential method for age estimation of difficult-to-age species such as the Baltic cod based on otolith microchemistry patterns. The objective of this study is to explore the applicability of otolith microchemistry patterns from the core to the edge of the otolith for age estimation, using samples from hydrographically contrasting ecosystems, in a three-step approach: (i) *concept development*: where patterns of opacity and elements from western Baltic cod with strong contrast between growth zones, good readability, and high confidence in age readings are used to identify elements with signatures matching growth zones with respect to maxima and minima at the same distances from the otolith core; (ii) *concept testing*: where the synchrony between the selected elements and opacity signals are examined on selected otoliths from the North Sea and eastern Baltic Sea cod with sufficient contrast between growth zones to provide reliable age estimates, and where the relationship of element incorporation across environments is tested; and (iii) *concept application*: where this approach is tested on eastern Baltic cod otoliths where contrast between growth zones is so limited that age estimation is not possible.

The intention with this paper is to demonstrate the potential for this approach with respect to concept and statistical analyses to encourage testing the hypotheses on other stocks and species.

Material and methods

Sample selection and preparation

Adult cod were sampled in the North Sea and the eastern and western Baltic Sea in winter/spring 1998. These samples were subsequently used to evaluate the suitability of elemental fingerprints for stock separation, for further details, see Heidemann *et al.* (2012). The age and readability ("good" or "bad") of each individual was estimated by two expert age readers. From these samples, a subset of otoliths was selected for the present study: Samples were grouped into two groups according to readability of their annual growth zones. From the "good" readability group only 4-year-old individuals were selected for comparability between areas (except for the eastern Baltic Sea where also a group of 3-year-old cod were selected), resulting in a total of $n = 34$ individuals (Table 1). Additionally, $n = 4$ individuals from the eastern Baltic Sea with similar size and "bad" readability preventing age estimation were selected for demonstrating the concept applicability. These individuals were (i) 49 cm male, (ii) 50 cm female, (iii) 51 cm male, and (iv) 48 cm female. Sample locations are shown in Fig 1. All individuals from the eastern and western Baltic Sea were adults in spawning or spent condition at capture. No information on maturity was available for the North Sea samples, but as their lengths were larger than the L_{50} maturity ogive for the North Sea (ICES, 2014b), they were assumed to be adult individuals too.

Otoliths were embedded in GTS polyester resin with MEKP hardener (Voss Chemie®) and sectioned transversely through the core using a semi-automatic sawing machine with diamond wafering blade (Conrad®). The sections were mounted on microscope slides with thermoplastic resin (Crystalbond 509) and polished with 3 μm lapping film (3M®).

Microchemical analyses

Otolith element composition was analysed along the longest growth axis from the core to the dorsal edge by laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) using a NewWave UP193 solid-state laser coupled to a ThermoFinnigan Element2™. Laser spot size was 75 μm, scan speed 4 μm s^{-1}, and pulse rate and irradiance were set to 10 Hz and ~1 GW cm^{-2}, respectively. Helium (0.4 l min^{-1}) was used as sample gas and argon (0.8 l min^{-1}) as make-up gas. Plasma power was 1200 W. Blanks were measured for 20 s before ablation. External calibration was performed by measuring NIST612 standard reference material after each transect (standard bracketing). Precision of measurements according to standard bracketing is specified in the last column of Table 2. The deviation in measurements of NIST612 from published values (Pearce *et al.*, 1997) was used for recalibrating the values for each element. It must be pointed out that the manganese concentration of 77.4 μg g^{-1} in NIST612 provided by Pearce *et al.* (1997) significantly differs from the newly determined value of 68 μg g^{-1} (Jochum *et al.*, 2011). The accuracy for Mg improves

Table 1. Overview of atlantic cod (*G. morhua*) samples used in this study.

Stock	Readability	Age	*N*	Size range
Western Baltic Sea	Good	4	12	56 (51–60)
North Sea	Good	4	8	36 (30–49)
Eastern Baltic Sea	Good	3	6	49 (46–52)
	Good	4	8	54 (47–67)
	Bad	?	4	49.5 (48–51)

Size values are averages with size range in brackets.

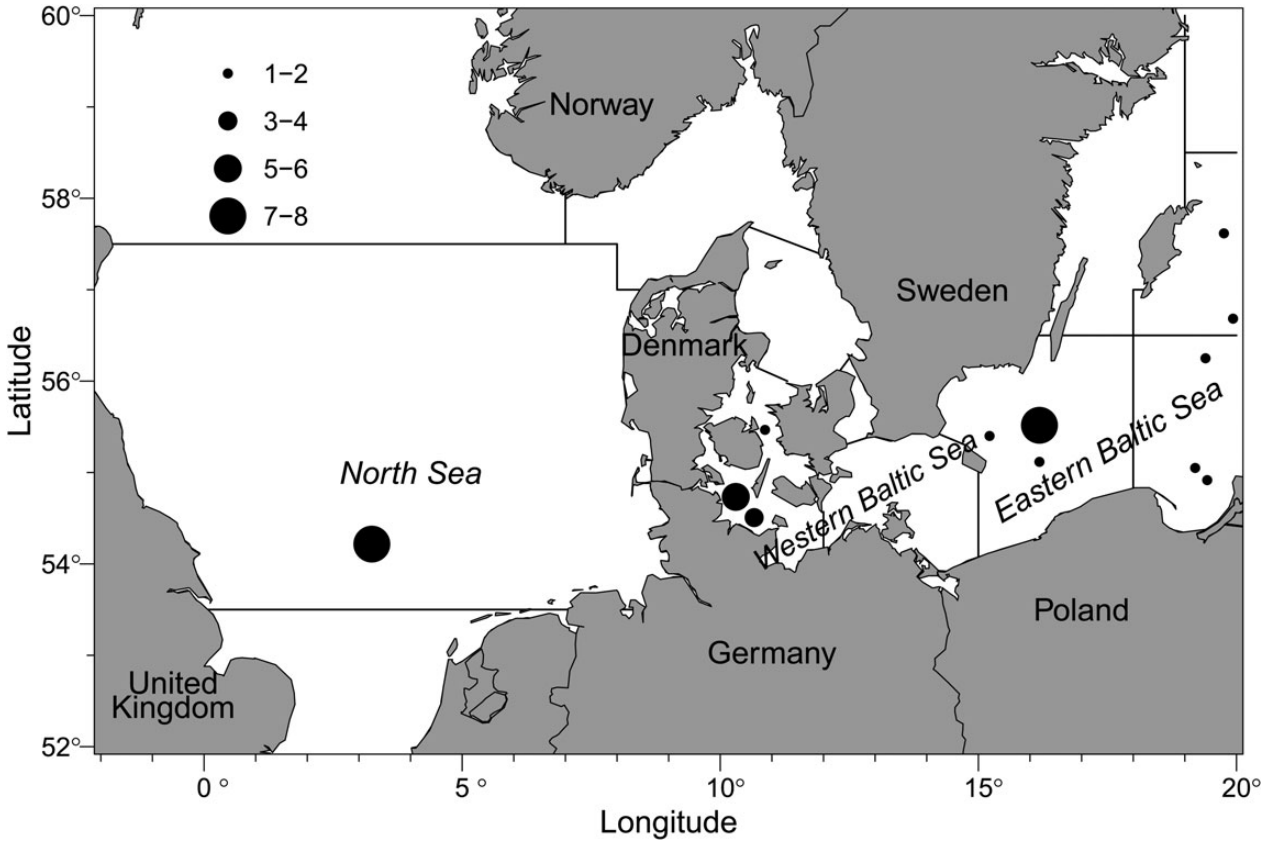

Figure 1. Map of the locations where Atlantic cod (*G. morhua*) were sampled. Symbol size represents number of individuals from the given location.

Table 2. Mean (± s.d.) element/calcium ratios of adult Atlantic cod (*G. morhua*) otoliths from different sampling areas and precision of measurements as relative standard deviation (rel. s.d.) of standard bracketing values from published values of NIST612 standard reference material according to Pearce *et al.* (1997)

Element	Units	NS	WBS	EBS	rel. s.d. [%]
Mg	μmol mol^{-1}	36.56 (11.14)	31.99 (11.02)	31.67 (10.56)	10.10
Mn	μmol mol^{-1}	5.66 (8.34)	19.82 (15.57)	13.53 (11.13)	5.76
Cu	μmol mol^{-1}	0.52 (0.88)	1.87 (2.58)	0.84 (1.49)	10.27
Zn	μmol mol^{-1}	1.15 (1.74)	2.33 (3.16)	0.95 (1.47)	16.17
Rb	μmol mol^{-1}	0.20 (0.12)	0.39 (0.28)	0.26 (0.19)	8.41
Sr	mmol mol^{-1}	2.05 (0.52)	1.79 (0.49)	1.44 (0.35)	4.98
Ba	μmol mol^{-1}	2.87 (2.10)	7.11 (4.60)	8.99 (5.28)	9.33
Pb	μmol mol^{-1}	0.03 (0.08)	0.17 (0.38)	0.06 (0.17)	18.30

NS, North Sea; WBS, Western Baltic Sea; EBS, Eastern Baltic Sea.

significantly if the Jochum *et al.* (2011) value rather than the Pearce *et al.* (1997) value for NIST612 is used for calibration. For internal standard, calcium was used with an assumed concentration of 38.8 wt% for the otoliths, which is similar to the NIES22 otolith standard reference material according to Yoshinaga *et al.* (2000). A pre-ablation was carried out before every measurement at 100 μm s^{-1} scan speed and a spot size of 120 μm to clean surface of the otolith material. More details about the analytical setup and data quality are described in Heidemann *et al.* (2012).

For data quantification, the Cetac GeoPro^{TM} software was used. Concentrations of the following elements were analysed: ^{7}Li, ^{23}Na, ^{25}Mg, ^{43}Ca, ^{55}Mn, ^{65}Cu, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{93}Nb, ^{111}Cd, ^{138}Ba, ^{208}Pb, and ^{238}U and converted to element/calcium ratios (μmol mol^{-1}) following standard practice. After an initial screening of all elements, we focus on eight candidate elements ^{25}Mg, ^{55}Mn, ^{65}Cu, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{138}Ba, and ^{208}Pb as concentrations of the other elements were either very low or highly variable between individuals. In the following, they will be referred to without their superscript. A summary of element concentrations may be found in Table 2.

Many of the otoliths used in these analyses sustained cracks during sectioning, resulting in small interruptions of the otolith profiles by intrusions of polyester resin. The element composition of the resin differs substantially from that of the otoliths, and measurement values attributable to the resin could therefore easily be removed.

To test whether the incorporation of the different elements is regulated by the same mechanisms across all three areas, correlation between element concentrations was tested by pairwise comparison of all elements using linear mixed effects model with element concentrations as dependent variable, geographic area as fixed effect, and individual fish as random grouping effect. *Post hoc* comparisons of the best model fit were carried out using Tukey contrasts for multiple comparisons of means. All statistical tests were carried out using R (R Development Core Team, 2009).

Otolith opacity

Otolith sections were viewed under a stereomicroscope (Leica MZ6) at a magnification of 2.8 μm pixel^{-1} using reflected light with a circular light source. Images were digitized using a standard set-up (8 bit/channel with a frame of 2048 × 1536 pixels, exposure 110 ms). The otoliths' visual appearance was then captured as light intensity values along a profile from centre to dorsal edge (next to the laser trench) using image analysis software ImageJ (ver. 1.48). Light intensity values range between 0 (black) and 255 (pure white). An annulus appears as "dark" owing to its lower opacity, with associated lower light intensity values. For each otolith, profiles of elements and opacity were thus obtained as a function of distance to the core. On each image, two expert age readers annotated which structures were identified and counted as annuli along the same axis as the profiles were measured. The two age readers' interpretations agreed in all otoliths with "good" readability. The distances from the otolith core to the annotated annuli were measured.

Profile analysis

Fish and their otoliths grow at different rates, resulting in otolith sizes that differ between individuals of the same age. To compare values between individuals, the profiles of individual elements and opacity were standardized by dividing the distance to the otolith core of each measurement by the total profile length. The distances to the annotated annuli were standardized therefore. In the following, all measurements are represented as a function of this relative distance ranging between 0 (core) and 1 (edge). Similarly, element concentrations vary considerably between individuals. To compare element concentrations across individuals, relative element values were calculated by dividing each measurement by the mean concentration, averaged over the entire profile.

Profiles of each individual otolith and element were then smoothed using Loess smoothing with a span (degree of smoothing) of 0.2 and a degree of polynomials of 2 (R Development Core Team, 2009). The smoothed profile values were then averaged across individuals with respect to relative distance from the core. In the following figures, these average values are shown $\pm$ s.d.

To find statistically significant (local) maxima and minima in the Loess smoothed profiles, we used the finite-differencing method in combination with a structural break model. Finite difference methods are methods to numerically solve differential equations, where finite differences are used to approximate the derivative of a function and difference equations to approximate differential equations (for details of method description, see Smith, 1985; Strikwerda, 2004; Morton and Mayers, 2005). Hence, the finite difference method relies on discretizing a function on a grid. In case of piecewise linear functions, finite differences form exactly the slopes of the piecewise segments. Given this, an internal point of an interval is a (local) maximum when a line segment with positive slope is followed by a line segment with a negative slope, in case of minima vice versa. In SAS/IML, we used the DIF function to compute finite differences and the SIGN function to generate a vector of signs ($+1$, 0, or -1) for a set of negative, positive, or zero slopes.

To indicate statistically significant local minima of the element profiles, we (i) identified the local maxima using the finite differencing method, and (ii) statistically tested the local minima contained in the between-maxima segments for significance based on a "valley"-type break template. To conceptualize this, we used a structural break model (Gröger *et al.*, 2011) where the break has been designed to approximate a valley-type segment. The resulting number of significant between-maxima segments is hence smaller or equal to the number of all segments identified. Only the four individuals used for "concept application" were used for testing the statistical significance of profile minima.

Results

Concept development

The cross sections of western Baltic cod otoliths have characteristic visual patterns with high contrast between growth zones (Figure 2a). Growth zones are formed simultaneously in all individuals, leading to well-defined opacity patterns even when averaging over 10 individuals (Figure 2b). Consequently, also the expert reader identified annuli occur within rather narrow intervals. The decreasing opacity towards the edge occurs in all otoliths and is attributable to the lower protein incorporation rates with fish size (Morales-Nin, 1986). The profiles of these 4-year-old cod show three well-defined zones with minima in opacity corresponding to the visually identified annuli. Owing to the general decrease in opacity and its position close to the edge of the otolith, the fourth minimum is, although readily observed in all otoliths, not clearly defined when profiles are averaged over several individuals. These otoliths also show consistent element signals, where the patterns of Cu, Zn, Rb, and to a lesser degree Pb are synchronous with the opacity profiles, while Mg and Mn show an inverse relationship with opacity (Figure 2b). For Ba and Sr, no patterns consistent with the opacity profiles were found. These results suggest that patterns of Cu, Zn, Rb, Mg, and Mn can potentially be used to estimate the correct age of cod in the western Baltic Sea. The ranges of annulus annotations are marked with horizontal lines in this and all subsequent figures.

Concept testing

The applicability of element patterns for age interpretation was tested on otoliths with clearly defined growth zones from the North Sea and with sufficiently discrete growth zones from the eastern Baltic Sea.

North Sea

In North Sea cod, otoliths show strong seasonality in opacity pattern formation (Figure 3a) with the same good correspondence in opacity minima with reader identified annuli and decrease of opacity towards the edge of the otolith. Virtually, all otoliths show signs of cracks with extruding embedding resin, which causes the patterns averaged over all individuals to be somewhat less clearly pronounced than in western Baltic cod. Nevertheless, these 4-year-old otoliths show generally the same relationship in element patterns with opacity as the western Baltic cod, with synchronous patterns in Cu, Zn, Rb, and Pb and inverse patterns in Mg and Mn. Contrary to the western Baltic samples, patterns of Sr show maxima in the range of reader identified annuli (Figure 3b).

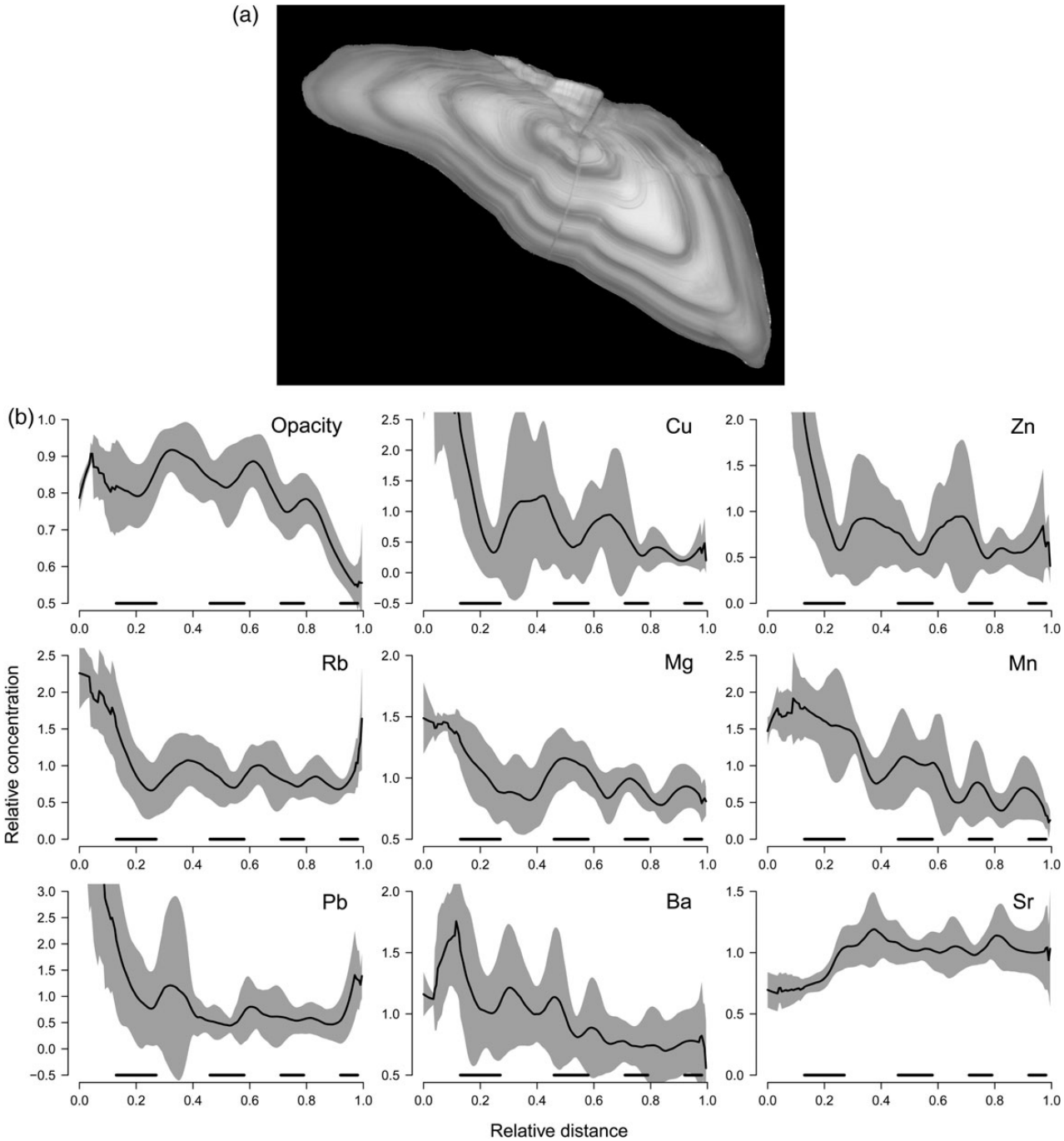

Figure 2. Western Baltic cod (*G. morhua*): (a) image of an otolith cross section from a typical 4-year-old cod, (b) profiles of opacity, Cu, Zn, Rb, Mg, Mn, Pb, Ba, and Sr (mean $\pm$ s.d., $n = 10$). Horizontal bars represent the location of the expert reader identified annuli.

Eastern Baltic cod

In eastern Baltic cod, two age classes were available. In this stock, contrast between growth zones is generally low and growth patterns characterized by large inter-individual variability (Hüssy *et al.*, 2010). The first annulus is generally not clearly defined in the axis used for profile measurements. Consequently, there is no minimum in opacity during first annulus formation, and rather weakly defined seasonal patterns in the average opacity values in both 3- (Figure 4a) and 4-year-old cod (Figure 5a). In both age classes, the same patterns in Cu, Zn, Rb, and Pb as in western Baltic cod and North Sea otoliths are evident with average profile minima corresponding to the range of expert reader identified annuli, particularly in the 3-year-old samples (Figure 4b and b). As in the North Sea and western Baltic Sea, Mg shows a pattern with maxima occurring in the range of these annuli—except the first minima in Cu, Zn, Rb, where Mg is also at a minimum. Contrary to the former samples, the signal of Mn is less well defined in eastern Baltic cod, while the Pb pattern is synchronous with Zn. As in the North Sea samples, the 4-year-old samples suffer from cracks throughout the otolith with extruding embedding

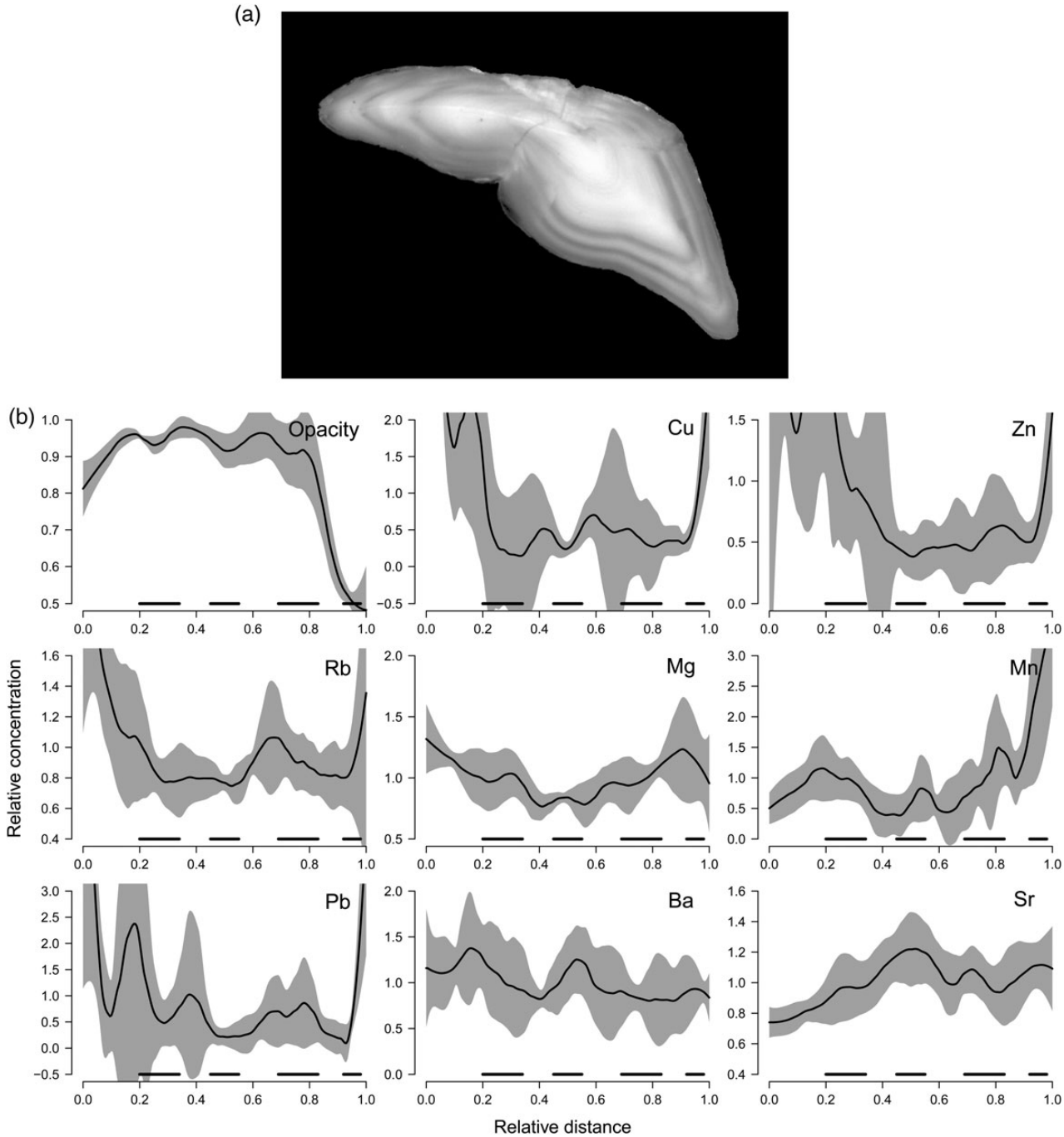

Figure 3. North Sea cod (*G. morhua*): (a) image of an otolith cross section from a typical 4-year-old cod, (b) profiles of opacity, Cu, Zn, Rb, Mg, Mn, Pb, Ba, and Sr (mean $\pm$ s.d., $n = 5$). Horizontal bars represent the location of the expert reader identified annuli.

resin, leading to less well-defined average values, although individual signals are much clearer.

These results confirm that the patterns of Cu, Zn, Rb, and Mg exhibit seasonal signals and may therefore be suitable as age estimation tool.

Correlation between element concentrations

To test whether the incorporation of the different elements is in fact regulated by a general physiological mechanism across geographic areas, pairwise comparison of the measured element concentrations (element/calcium ratios, not the standardized relative values) and otolith opacity was carried out. Opacity was not related with any of the elements, while most element concentrations were to a varying degree correlated with each other (Table 3). Pearson product moment correlation coefficients between the different elements and opacity are shown in Table 3, together with the ANCOVA statistics. No significant effect of geographic area was found for Cu, Zn, and Rb (LME, all area effects >0.05). In all analyses with the elements Mg and Mn, the samples from the North Sea differed significantly from the two Baltic Sea areas, while the samples from the

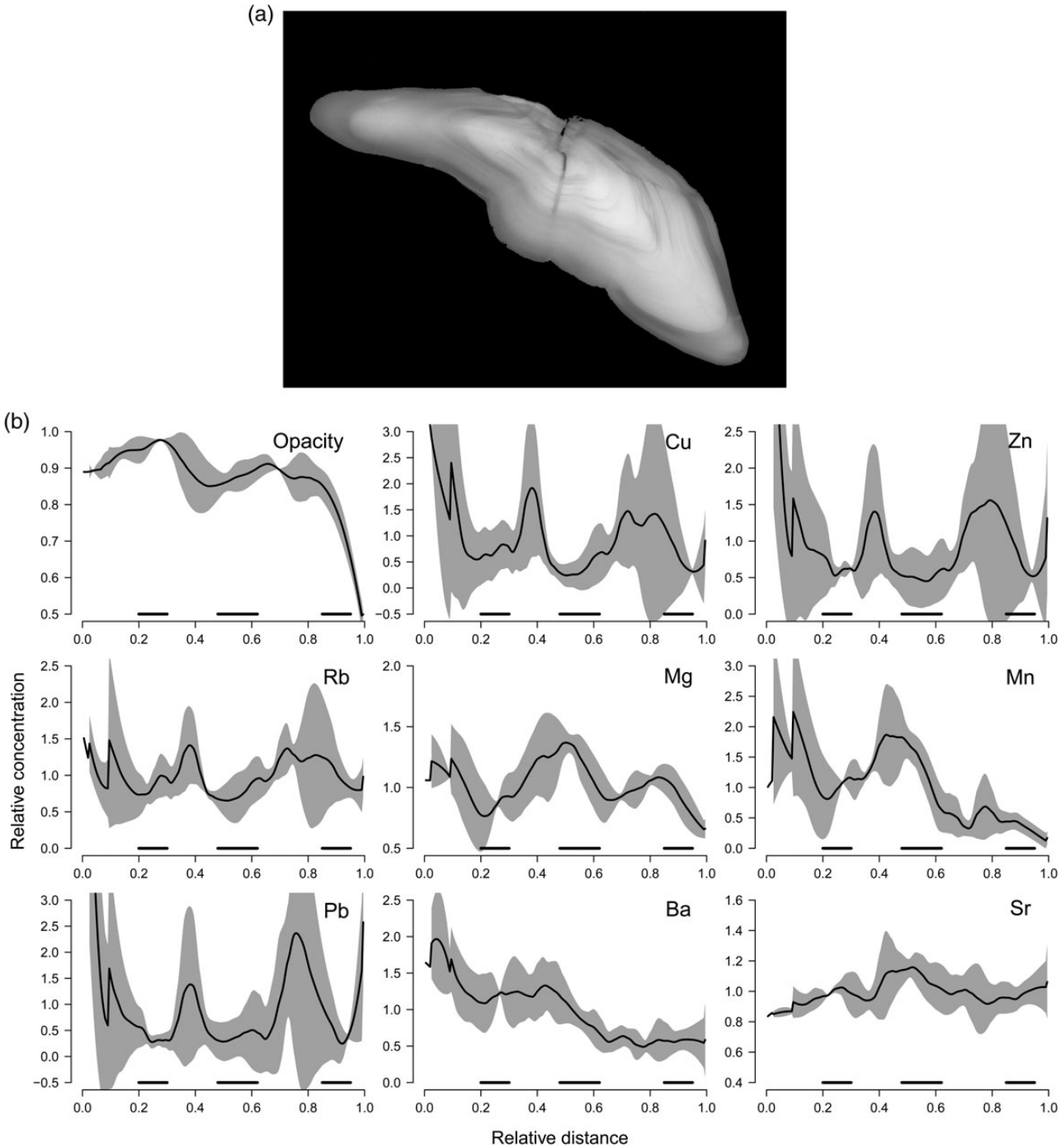

Figure 4. Eastern Baltic cod (*G. morhua*), age = 3 years: (a) image of a typical otolith cross section, (b) profiles of opacity, Cu, Zn, Rb, Mg, Mn, Pb, Ba, and Sr (mean ± s.d., $n = 3$). Horizontal bars represent the location of the expert reader identified annuli.

western Baltic Sea differed from the other two areas in analyses with the elements Sr, Ba, and Pb (Tukey contrasts, $p < 0.05$).

Concept application

In the following, examples of four eastern Baltic cod are shown where the insight gained from the above results and the statistical identification of element profile minima is used to estimate their age (Figure 6). The visual appearance of these otoliths is characterized by low contrast between zones and narrow zones that may not represent annuli. Expert age readers assigned these otoliths' readability as "bad" in that they were not able to estimate the fish's accurate age (Figure 6a–d). The uniform opacity profiles support this interpretation (thin grey lines in Figure 6e–h). In all four otoliths, the element signals of Cu, Zn, Rb, and Pb show a clear pattern with minima spaced at intervals very similar to what one would expect of annuli (Figure 6e–h). As the four elements exhibit similar patterns, the significance of minima was only tested for the Cu profiles. The location of each of the statistically significant

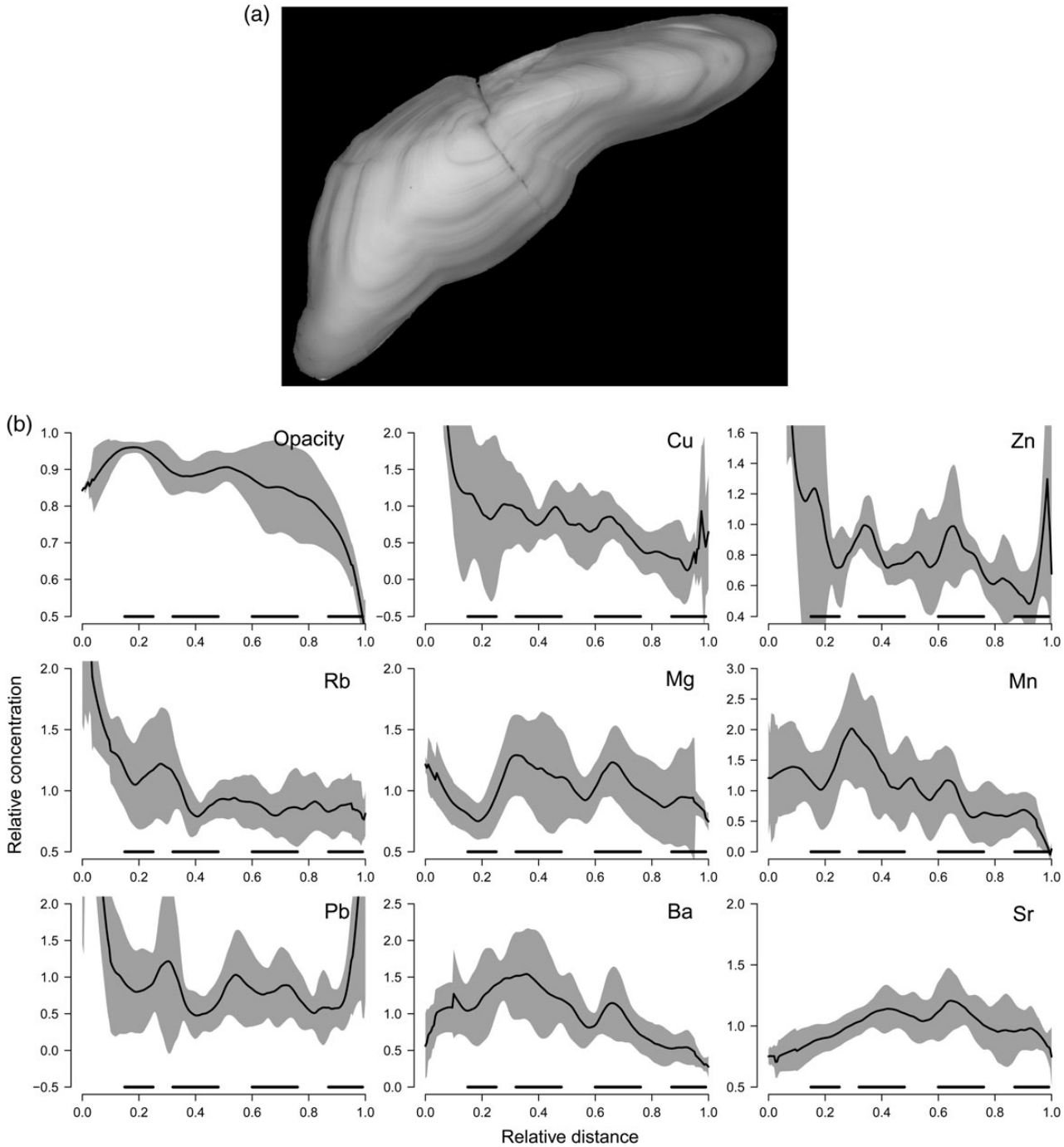

Figure 5. Eastern Baltic cod (*G. morhua*), age = 4 years: (a) image of a typical otolith cross section, (b) profiles of opacity, Cu, Zn, Rb, Mg, Mn, Pb, Ba, and Sr (mean ± s.d., $n = 7$). Horizontal bars represent the location of the expert reader identified annuli.

local profile minima are indicated with horizontal bars underneath the profiles for each individual (Figure 6e–h). Mg profiles in these individuals were also analysed and show statistically significant maxima concurrent with the minima in Cu, Zn, Rb, and Pb, but the signals are not very strong and are therefore not shown in the figures. The element signals identified these otoliths to be from fish of 7 years and 49 cm (Figure 6e), 6 years and 50 cm (Figure 6f), 6 years and 51 cm (Figure 6 g), and 6 years and 48 cm (Figure 6 h).

Discussion

For otolith microchemistry to serve as useful tool for age validation the mechanisms of element incorporation has to be a function of seasonal patterns in either environment and/or growth and consistent across age classes and years. For general applicability, the incorporation mechanism also needs to be generic across stocks. In the present study, the usefulness of Mg, Mn, Cu, Zn, Rb, Sr, Ba, and Pb was tested using otolith samples from three hydrographically contrasting ecosystems. The North Sea is characterized by high

Table 3. ANCOVA table of pairwise comparison of atlantic cod (*G. morhua*) otolith element concentrations (*E*) and the area effect on intercept (*A*) and slope (*E:A*) of that relationship.

								Area effect	
	Elements	**Intercept**	**Slope**	**d.f.**	***F***	**Corr**	p_E	p_A	$p_{E:A}$
Opacity	Mg	133.31	0.03	10 641	3.25	0.02	n.s.	n.s.	n.s.
	Mn	136.85	−0.77	10 641	140.2	−0.11	n.s.	n.s.	n.s.
	Cu	134.13	0.27	10 641	6.83	0.03	n.s.	n.s.	n.s.
	Zn	134.74	−0.17	10 641	4.04	−0.02	n.s.	n.s.	n.s.
	Rb	133.59	3.001	10 641	10.86	0.03	n.s.	n.s.	n.s.
	Sr	137.80	−0.02	10 641	20.49	−0.04	n.s.	***	*[a]
	Ba	131.92	0.42	10 641	91.42	0.09	n.s.	n.s.	n.s.
	Pb	135.69	−12.46	10 641	271.8	−0.16	n.s.	n.s.	n.s.
Mg	Mn	31.76	0.13	10 461	318.4	0.17	*	***	ns[a]
	Cu	31.34	−1.92	10 461	1425	−0.34	**	n.s.	***[a]
	Zn	30.89	−1.66	10 461	1715	−0.37	**	***	ns[a]
	Rb	30.11	−12.004	10 461	1412	−0.25	**	**	ns[a]
	Sr	43.83	−0.006	10 461	810.9	−0.27	*	***	ns[b]
	Ba	31.44	0.36	10 461	255	0.15	*	**	ns[a]
	Pb	32.80	8.04	10 461	439.8	0.20	*	**	**[a]
Mn	Cu	11.41	−1.69	10 461	647.6	−0.24	*	***	*[a]
	Zn	11.43	−1.19	10 461	513.3	−0.21	*	***	ns[a]
	Rb	9.14	−14.59	10 461	671.9	−0.24	*	***	ns[a]
	Sr	25.68	−0.007	10 461	749.1	−0.26	**	***	*[c]
	Ba	7.60	0.97	10 461	1287	0.33	**	***	*[b]
	Pb	12.65	7.33	10 461	230.8	0.15	n.s.	***	ns[a]
Cu	Zn	0.098	0.66	10 461	2.72×10^4	0.82	***	n.s.	n.s.
	Rb	−0.689	6.42	10 461	1.34×10^4	0.75	***	n.s.	n.s.
	Sr	2.956	−0.001	10 461	1153	−0.31	*	***	***[c]
	Ba	0.341	0.14	10 461	1301	−0.33	*	***	***[c]
	Pb	0.743	−4.35	10 461	6072	−0.60	**	***	***[c]
Zn	Rb	−0.418	7.033	10 461	7998	0.65	***	n.s.	n.s.
	Sr	3.536	−0.001	10 461	1015	−0.30	*	**	***[c]
	Ba	0.823	−0.134	10 461	729.5	−0.25	*	n.s.	*[c]
	Pb	1.001	−6.230	10 461	9698	−0.69	**	**	***[c]
Rb	Sr	0.521	−0.0001	10 461	966.3	−0.29	*	***	**[c]
	Ba	0.203	0.014	10 461	1020	0.30	*	***	*[c]
	Pb	0.248	−0.425	10 461	3645	−0.51	**	**	*[c]
Sr	Ba	1928.638	−18.620	10 461	302.4	−0.17	*	***	*[d]
	Pb	1857.742	−402.432	10 461	495.9	−0.21	*	***	**[d]
Ba	Pb	5.615	3.249	10 461	402.1	0.19	*	***	*[b]

Correlation between elements given as Pearson product moment correlation, superscripts denote which areas differ from each other.
n.s., not significant.
aNorth Sea differs from the eastern and western Baltic Sea.
[b]All stocks differ from each other.
[c]Western Baltic Sea differs from North Sea and eastern Baltic Sea.
[d]Eastern Baltic Sea differs from North Sea and western Baltic Sea.
Significance levels: ***$p < 0.001$; **$p < 0.01$, *$p < 0.05$.

salinity and pronounced seasonal temperature signals, while the eastern Baltic Sea is a brackish environment with distinct vertical stratification of the water column with respect to temperature and salinity. In the western Baltic Sea, the transition area between Baltic Sea and Kattegat, hydrographic conditions are extremely variable and strongly dependent on meteorological forcing (Schinke and Matthäus, 1998).

This study found distinct periodic patterns in the concentration of Cu, Zn, and Rb from the core to the edge of the otolith that co-varied with otolith opacity, with highest element incorporation during the summer growth season. These patterns formed consistently across age classes and geographic area, strongest in the western Baltic, and weakest in the North Sea. In the eastern Baltic Sea, Pb patterns also co-varied consistently with opacity. Conversely, the concentrations of Mg and Mn apparently followed an inverse pattern with opacity with maxima in element concentration during winter zone formation. Strong correlations between element concentrations of Cu, Zn, and Rb without differences between geographic areas suggest that the incorporation mechanisms of these elements do not exclusively depend on environmental concentration, which can be expected to differ between these areas, but regulated by similar mechanisms (Campana, 1999).

The pathway of elements from the environment into the otolith depends on a combination of ambient element concentration, their uptake through gills or intestine into the blood plasma, across the endolymphatic epithelium, and onto the surface of the otolith (Payan *et al.*, 2004) where each process acts as a barrier altering the rate at which elements are transferred (Campana, 1999). Elements such as Cu, Zn, and Pb generally are bound to proteins and their availability is thus easily affected by physiological fractionation in the body (Watanabe *et al.*, 1997). Elements like Li, Mg, Sr, Ca, and Ba on the other hand, typically occur as free ions in both

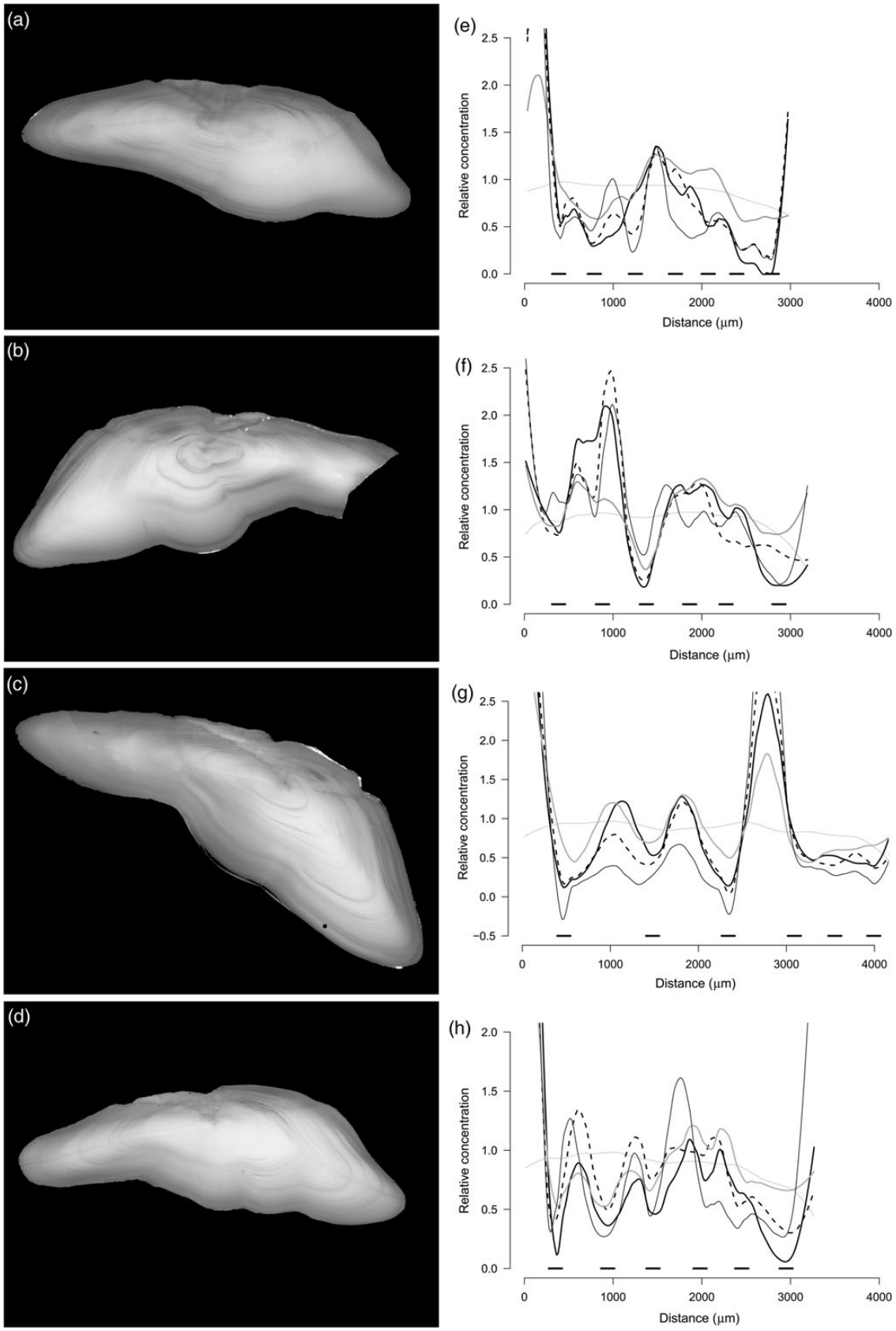

Figure 6. Examples of eastern Baltic cod (*G. morhua*) otolith cross sections that do not allow traditional age reading (a – d) and their corresponding elemental profiles in Cu (solid black), Zn (broken black), Rb (solid grey), and Pb (thin solid black) as a function of absolute distance to the centre of the otolith (e – h). Also shown are the opacity profiles (thin solid grey). Horizontal bars indicate the statistically significant profile minima. Estimated ages of the four otoliths are: (e) 7 years (49 cm), (f) 6 years (50 cm), (g) 6 years (51 cm), and (h) 6 years (48 cm).

seawater and blood plasma and their passage from environment to otolith is thus less likely to be affected by blood protein chemistry (Kalish, 1991; Sturrock *et al.*, 2014). Consequently, incorporation of elements into the otolith may be subject to environmental or physiological control. The essential question in relation to this study is: Do the element signals found in this study represent seasonal variations in environmental condition and/or physiological processes that are consistent between years and over the entire life of a fish? In elements under environmental control, incorporation into the otolith is correlated with ambient concentrations and/or temperature, whereas in elements under physiological control incorporation is linked to processes like growth, reproduction, ontogeny, and age (Kalish, 1991; Walther *et al.*, 2010; Sturrock *et al.*, 2012, and references therein). However, the incorporation mechanisms of specific elements into the otolith are still not fully understood, as documented by the often-opposing results (Sturrock *et al.*, 2012).

Elements under environmental control

Most studies agree that the incorporation of the elements Sr and Ba depends on ambient concentrations which are correlated with primarily salinity in marine environments (Kalish, 1989; Campana, 1999; Bath *et al.*, 2000; Milton and Chenery, 2001; Walther and Thorrold, 2006; Macdonald and Crook, 2010), presumably with an interaction between temperature and salinity for Sr (Kalish, 1989; Bath *et al.*, 2000; Elsdon and Gillanders, 2002; Miller, 2011). However, Sr incorporation may only be related to environmental concentrations in fresh and brackish water and less so in saltwater (Brown and Severin, 2009). The lack of a seasonal signal in Sr and Ba in the present study is therefore to be expected. Also Mn incorporation depends on ambient concentrations as a function of hypoxia (Elsdon and Gillanders, 2002; Limburg *et al.*, 2011; Mohan *et al.*, 2014) and potentially a strong temperature effect (Miller, 2009; Marohn *et al.*, 2011). The inverse relationship between opacity and Mn observed in the western Baltic and North Sea otoliths concurs with Mohan *et al.*'s (2014) results, while the frequently occurring hypoxia in the eastern Baltic (Limburg *et al.*, 2011, 2014) seems to override that temperature signal resulting in a more variable element signal. Also Pb is generally associated with pollution in the environment (Campana and Thorrold, 2001; Sturrock *et al.*, 2014). The strong signals observed in the eastern Baltic samples in the present study may thus reflect seasonal winter migrations from shallow towards greater depths (Oeberst, 2008).

Elements under physiological control

It is considerably more difficult to quantify the effect of physiological processes on element incorporation as it is difficult to separate for example the effect of temperature and growth rate, as growth is generally strongly influenced by temperature (Kalish, 1991; Bath *et al.*, 2000). One example is Mg, where Elsdon and Gillanders (2002) found a strong link between incorporation and temperature, but where Martin and Thorrold (2005) showed that this apparent temperature effect was actually attributable to fish growth and not temperature. Martin and Thorrold's (2005) study also supports the inverse synchrony of Mg and opacity observed in the present study. Some of the first studies of Cu and Zn incorporation found a significant link with ambient water concentrations (Milton and Chenery, 2001; Arai *et al.*, 2007). However, Miller *et al.* (2006) documented that otolith concentrations of both Cu and Zn are associated with the soluble part of the protein matrix. The soluble otolith protein incorporation is tightly linked with protein consumption and growth (Hüssy and Mosegaard, 2004) which in turn are subject to seasonal variation in subtropical areas (e.g. Daan, 1973). Laboratory experiments have confirmed the consumption-related Zn uptake into the otolith (Ranaldi and Gagnon, 2008). The link between Cu, Zn, and protein matrix thus provides a credible explanation for the seasonal signal in Zn concentration which co-varied with annual growth zones in salmonids (Halden *et al.*, 2000; Limburg and Elfman, 2010) and the patterns observed in this study.

Applicability of the approach

Before analysis of element profiles can be implemented as ageing tool, a number of caveats relating to biological and methodological aspects of the approach presented here need to be resolved.

Most elements highlighted in this study as potential indicators of age, Zn, Cu, and Mg, are essential elements in a multitude of physiological processes relating to growth, reproduction, development, vision, and immune function (Watanabe *et al.*, 1997). The influence of variations in particularly maturation cycle and growth therefore needs to be examined. Plasma concentrations of Zn and Cu, and thus their availability to transport into the endolymph (Sturrock *et al.*, 2014) are highly dependent on the reproductive cycle with lowest values during spawning in European plaice (Sturrock *et al.*, 2014). The onset of maturation and reproductive cycle may thus potentially alter the seasonality of element incorporation observed in juvenile fish. From the data presented here with minima in Cu and Zn occurring consistently during reader defined annuli, it is however neither evident to what extent the maturation cycle may have impacted on the element signals nor the direction of the effect (signal enhancement or attenuation). In this context, the present result may suffer from a methodological problem attributable to the relatively large laser-ablation spot size of 75 μm used. Since otolith growth rate decreases exponentially with time, this may have caused/contributed the observed attenuation of the element signals with otolith size/fish age (Halden *et al.*, 2000).

If an element's incorporation is influenced by the fish's growth rate, element signals may not be useful for age estimation if fluctuations in fish growth rate are not strictly annual (Campana, 1999). Halden *et al.* (2000) hypothesized that strong seasonal patterns in Zn, but with faster uptake during the first few years and declining concentration throughout life, were linked to food consumption. Pronounced seasonal patterns both with respect to prey species selection and quantity of food consumed are well documented for eastern Baltic cod (Bagge and Bay, 1987; Bagge *et al.*, 1994). While individual fish may deviate from strictly annual growth pattern, the average individual does seem to comply with a general seasonal pattern in consumption and growth, thereby resulting in the observed signals in averaged element profiles. The equally strong element signals in two individuals both estimated to be 5 years old, without visual contrast and of different size growth rates support the seasonal nature of these signals.

Why then are element signals in eastern Baltic cod much stronger than opacity profiles? Eastern Baltic cod differ from most other cod stock in that most these otoliths are characterized by very low contrast between growth zones or even an apparent absence of visually identifiable annuli (Hüssy, 2010). The otolith macrostructure of eastern Baltic cod often lacks strong seasonal variation in opacity owing to the unique combination of a variety of different abiotic and biotic factors (see Hüssy *et al.*, 2009), coupled with interacting seasonal variation in feeding intensity (Bagge and Bay, 1987; Bagge *et al.*, 1994), and particularly irregularly occurring vertical migrations between water masses of different temperature (Nielsen *et al.*, 2013). However, growth rates in the form of length mode

progression show pronounced seasonality (Oeberst, 2008). Since the elements like Cu and Zn are primarily under physiological control, it seems reasonable to assume that the observed elemental signals represent these seasonal growth patterns.

To our knowledge, this is the first study showing that even in otoliths with hardly any visible contrast between seasonal growth zones and consistent pattern formation, the element signals showed strong cyclical patterns with clearly defined minima and maxima. Analysis of element patterns from otolith core to edge may thus provide a tool for age estimation and/or validation in eastern Baltic cod and other fish species where traditional age reading is difficult. The two most important issues for the applicability of this approach to age estimation of fish are (i) development of a statistical approach for subjective signal interpretation and (ii) validation of the observed chemical signals' periodicity. Visual identification of profile minima suffers just as much from subjectivity as traditional age estimation. With the structural break method presented here, we have presented one possible solution. To avoid replacing one interpretation problem—traditional age reading—by another interpretation problem—chemical patterns—extensive validation of this approach is also necessary. To that end, analysis of known-age samples is mandatory. Tagging of individuals with subsequent release into the wild and later recapture provides such samples (i.e. de Pontual *et al.*, 2003). Ideally, external tagging of the cod should be accompanied by chemical marking of the otolith that does not interfere with the elements under evaluation, i.e. strontium chloride (i.e. Hüssy *et al.*, 2009). Other approaches that may serve as validation methods are the correlation of chemical signals with daily increment patterns in fish younger than 3 years (Hüssy, 2010) and the tracking of cohorts based on characteristic chemical signals deposited early in life (i.e. Upton *et al.*, 2012). In the Baltic Sea, periodic large-scale inflow events of saline Atlantic water are likely candidates for providing such signals (Schinke and Matthäus, 1998).

We hope that this study will encourage other researchers to test the general applicability of this approach not only on other cod stocks but also on different species from within the Baltic Sea and other ecosystems, including the development of the best analytical tools for pattern recognition.

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