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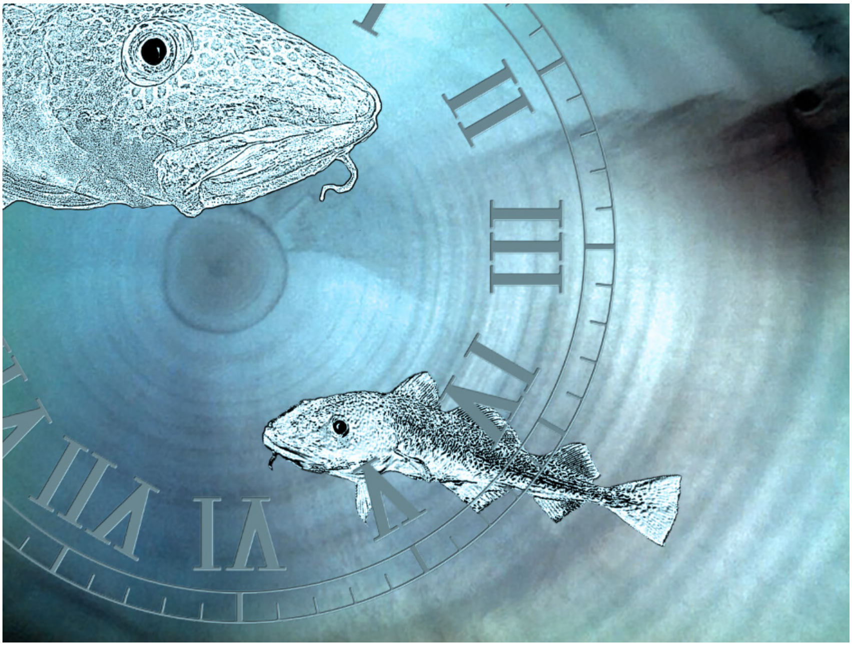
Losing track of time: causes and solutions for the problematic determination of Baltic cod age

Yvette Heimbrand



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Yvette Heimbrand
Faculty of Natural Resources and Agricultural Science
Department of Aquatic Resources
Öregrund

SWEDISH UNIVERSITY
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Abstract

Age is a fundamental parameter in biology and fishery science to estimate growth and population parameters. Losing track of time, in the sense of lacking reliable estimates of age and growth creates substantial problems for managing difficult-to-age-species. This thesis explores the use of fish otolith chemistry to improve age determination. Otoliths produce annual growth zones in the form of alternating opaque and translucent rings, corresponding to fast growth in summer and autumn and slow to no growth in winter. For the eastern Baltic cod (EBC), degraded environmental conditions led to a worsening of visual contrast in their otoliths. The EBC served as a model species to test new methods of aging, taking advantage of seasonally driven patterns in otolith chemical constituents. Experiments demonstrated that patterns of physiologically regulated elements significantly improved agreement among untrained chemical readers when compared to experienced readers using standard visual techniques, and that Mg:Ca and P:Ca showed the clearest patterns. Validations demonstrated mechanistic correlation with fish growth, condition, temperature, parasites and proximity to visible winter zones identified by disappearance of daily rings. EBC otoliths from the Stone Age to the present provided historical perspective. Mn:Ca was correlated with hypoxia and water temperature, indicating highest levels in the 2010s. Metabolic status, proxied by Mg:Ca was highest during the 1980s and 1990s, to thereafter decline. Predicted mean length at age by decade showed highest growth in the 1990s and a dramatic decline in the 2010s. The results from this thesis fill crucial knowledge gaps and provide current and historical insights on how EBC biology has changed over time that could improve the management of the threatened population.

Keywords: age validation, Baltic cod, otolith chemistry, Mg:Ca, P:Ca, hypoxia

Author's address: Yvette Heimbrand, SLU, Department of Aquatic Resources, Institute of Coastal Research, Skolgatan 6, 742 42 Öregrund, Sweden

Problematisk åldersbestämning av östersjötorsk, orsaker och en lovande lösning

Sammanfattning

Ålder är en grundläggande biologisk parameter inom fiskeriforskning. Information om ålder och tillväxt används som underlag vid beståndsuppskattning. Felaktig åldersuppskattning kan därför orsaka stora problem i fiskeriförvaltningen. Syftet med denna doktorsavhandling är att undersöka om kemianalyser av otoliter kan användas för att bestämma och validera ålder av fisk. Otoliter bildar under hela fiskens liv tillväxtzoner i form av opaka och translucenta ringar, som motsvarar snabb tillväxt på sommaren och hösten och långsam till ingen tillväxt på vintern. Dessa årsringar används för att åldersbestämma fisk. Torsk från det östra beståndet i Östersjön är svåra att åldersbestämma. Allt sämre tillväxt och vattenkvalitet har orsakat lägre kontrast mellan årsringarna som blivit otydliga och svårtolkade. Dessa otoliter användes för att testa en ny metod baserad på analyser av säsongsmässiga mönster av spårämnen i otoliter. Resultaten visade att mönstren i fysiologiskt reglerade spårämnen gav bättre överensstämmelse mellan åldersläsare av den nya kemiska metoden jämfört med den traditionella metoden och att magnesium och fosfor visade de tydligaste mönstren. Validering visade ett samband mellan dessa spårämnen och tillväxt, kroppskondition, temperatur och antalet leverparasiter. Det visade sig även att de kemiska mönstren överensstämde med motsvarande årsringar i otoliten som validerades med dagzonsanalys. Kemianalyser av torskotoliter från förra seklet och stenåldern gav ett historiskt perspektiv. Det fanns ett samband mellan mangan i otoliter, syrebrist och vattentemperatur, som indikerade att den största exponeringen för syrebrist hade skett under 2010-talet. Magnesium reflekterade metabolisk status och uppvisade högsta nivåer under 1980- och 1990-talet för att därefter minska. Uppskattad medellängd vid ålder uppvisade den högsta tillväxthastigheten på 1990-talet och en dramatisk nedgång under 2010-talet. Resultaten fyller viktiga kunskapsluckor om nutida och historiska förändringar i torskens biologi som kan användas som underlag för att förbättra förvaltningen.

Nyckelord: åldersvalidering, torsk, otolitkemi, magnesium, fosfor, Östersjön

Författarens adress: Yvette Heimbrand, SLU, Institutionen för Akvatiska Resurser, Kustlaboratoriet, Skolgatan 6, 742 42 Öregrund, Sverige

Dedication

To my family

Oh, a wonderful stream is the river of Time,
As it runs through the realm of tears,
With a faultless rhythm and a musical rhyme,
And a boundless sweep and a surge sublime,
As it blends with the ocean of Years.

- Benjamin Franklin Taylor, The Isle of Long Ago

Who hears the fishes when they cry?
It will not be forgotten by some memory that we were contemporaries.
- Henry David Thoreau

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Karin E. Limburg, Mark J. Wuenschel, Karin Hüssy, Yvette Heimbrand and Melvin Samson (2018) Making the otolith magnesium chemical calendar-clock tick: Plausible Mechanism and Empirical Evidence, Reviews in Fisheries Science & Aquaculture, 26:4, 479-493

II. Yvette Heimbrand, Karin E. Limburg, Karin Hüssy, Michele Casini, Rajlie Sjöberg, Anne-Marie Palmén Bratt, Svend-Erik Levinsky, Anastasia Karpushevskaia, Krzysztof Radtke and Jill Öhlund (2020). Seeking the true time: Exploring otolith chemistry as an age-determination tool. Journal of Fish Biology 97:2, pages 552-565

III. Yvette Heimbrand, Karin E. Limburg, Karin Hüssy, Maria Ovegård and Michele Casini (2021). Taking the lifelong pulse of a fish: Otolith chemical responses to growth and metabolic activity of Baltic cod. (manuscript)

IV. Yvette Heimbrand, Karin E. Limburg, Karin Hüssy, Michele Casini, Tomas Naeraa and Monica Mion (2021). Canaries in the Baltic Sea “coal mine”: Fish otoliths document accelerating climate impacts (manuscript)

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The contribution of Yvette Heimbrand to the papers included in this thesis was as follows:

I. Participated in planning and writing, conducted part of the laboratory work and otolith chemical analyses, coauthor of the published manuscript.

II. Led the planning, designing of the study and data collection. Conducted laboratory work, otolith chemical analyses and statistical analyses. Organized an international age calibration exercise with nine age readers in SmartDots, a new system for age calibrations developed by ICES. Handled the review process. Primary author of the published manuscript. I presented this study as an oral presentation at the 5th International Otolith Symposium in Keelung, Taiwan in 2018 and at the 5th International Sclerochronology Conference in Split, Croatia in 2019.

III. Planned and designed the study and data collection, conducted laboratory work, otolith chemical analyses and statistical analyses, primary author of the manuscript.

IV. Participated in planning, designing of the study and data collection, conducted laboratory work, otolith chemical analyses and statistical analyses, primary author of the manuscript. I presented this study as an oral presentation at the Baltic Sea Science Congress in 2019 in Stockholm, Sweden.

1. Introduction

1.1 Losing track of time

Nature is a realm of change, encompassed by time, with biological rhythms and ecological interactions directed by seasons and cycles of the moon and tide under influence by the transient light between dusk and dawn. Time leaves traces, layers we can unfold and measure, such as vertical time-series of sedimentary rock and fossil tree rings serving as calendars for the archaeologist.

In temperate teleost fishes, lifelong production of alternating opaque and translucent growth zones in the otoliths, calcium carbonate structures in the inner ear balance and hearing system (Pannella, 1971; Popper *et al.*, 2005) creates individual time capsules, storing information of past environmental conditions experienced by each and every fish (Campana, 1999; Sturrock *et al.*, 2012).

In fisheries management, age and growth are key biological parameters for understanding the status of a population (ICES, 2014). The size and age when the fish become sexually mature in relation to growth rate and lifespan are fundamental parameters when assessing the links between recruitment and fishing mortality and predicting spawning stock biomass and reproduction potential (Hutchings, 2002).

Traditionally, fish ages are determined by counting the number of annual growth zones in otoliths or other calcified structures such as scales, operculum bones, vertebrae, or fin rays (Campana and Thorrold, 2001). For the Atlantic cod (*Gadus morhua* L. 1758) in the eastern Baltic Sea however, the interpretation of seasonal zonation has proven increasingly more complicated over time due to low contrasting structures and irregular growth patterns (Hüssy, 2010). In 2014, an international age calibration exercise revealed such substantial disagreement among age readers and low precision that the routine method was considered too uncertain and was abandoned

(Hüssy *et al.*, 2016b). The resulting lack of reliable biological parameters contributed to a failed analytical age-based stock assessment (ICES, 2014). Consequently, finding new solutions for age estimating eastern Baltic cod became a priority.

1.2 Baltic Sea

The Baltic Sea region was historically formed by processes of isostatic land uplift and eustatic sea-level rise following the latest deglaciation 15 000 years ago (Björck, 2008). As the connections to the ocean transformed over time, the Baltic Sea experienced altering phases of sea level and salinity. The melting Scandinavian ice sheet initially created the freshwater Baltic Ice Lake, but as the ice coverage continued to retreat, new links to the Atlantic Ocean opened up, forming the Yoldia Sea around 10 000 years ago (Björck, 1995).

The speed of post-glacial isostatic rebound exceeded rising sea level, and so the rising land mass closed the connection during the Ancylus Lake freshwater phase (Björck and Dennegård, 1988). With increased global sea level rise around 9800 years ago, new pathways in the southwest opened up once more, initiating the brackish Littorina Sea transgression and the Baltic Sea has since then been connected to marine waters (Andrén *et al.*, 2011; Björck, 2008).

The Holocene Thermal Maximum 8000–5000 years ago created a warmer climate, and from palaeoenvironmental geochemical and biological proxies it is evident that the saline Littorina was a very productive sea (Weckström *et al.*, 2017). During the Neolithic time period 4500 – 5000 years ago, salinity was higher with warmer summers and colder winters than today (Gustafsson and Westman, 2002; Olson, 2008). Today, the semi-enclosed, non-tidal Baltic Sea (Fig. 1) in northern Europe is one of the largest brackish seas in the world, with a third of the area being shallower than 30 m.

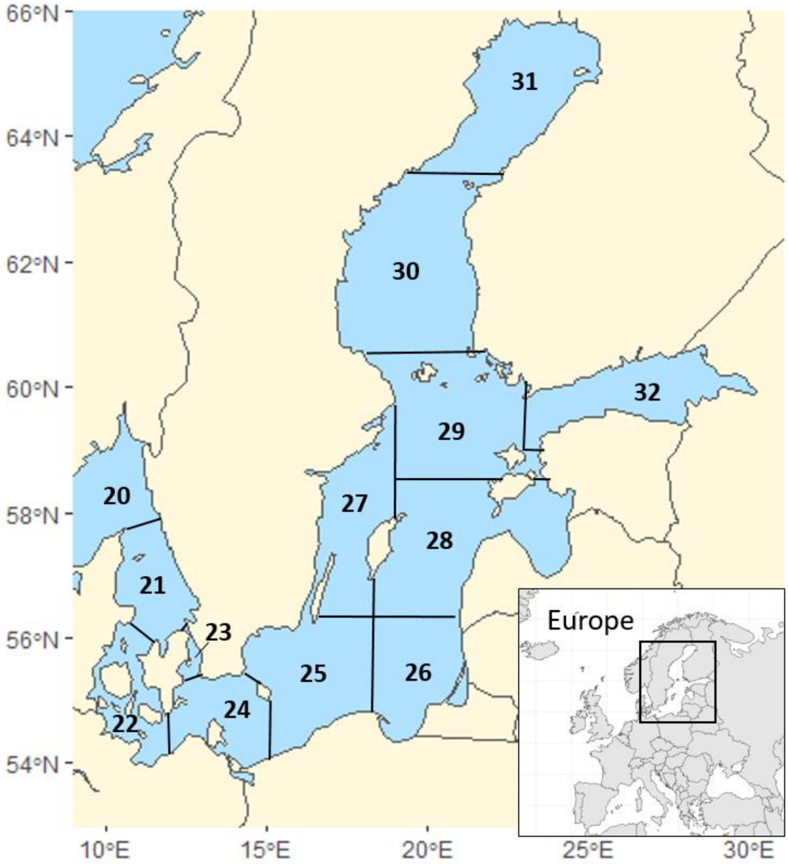

Figure 1. Map of the Baltic Sea with the International Council for the Exploration of the Sea's statistical subdivisions (ICES SDs) separated by black lines. The eastern Baltic Sea consists of SD 25 – 32.

Strong hydrological influence from the large watershed drainage area, around four times the size of the surface area (HELCOM, 2017), dictates much of the physical dynamics, similar to an outsized estuary. The narrow and shallow connections to the Atlantic Ocean limit the inflow of saline oxygenated water and ventilation of the stratified bottom layers occur with less frequency and intensity now than before (Mohrholz *et al.*, 2015).

The irregular inflow slows down the water exchange, contributing to the long turnover time of up to 30 years (Kullenberg and Jacobsen, 1981;

Reissmann *et al.*, 2009). The salinity gradient ranges from close to fresh water in the Bothnian Bay in the north to around 8 PSU and in the central Baltic Proper and between 10 and 20 PSU in the south (Kullenberg and Jacobsen, 1981). The permanent halocline vertically stratifies and separates the water column into colder, higher salinity water at depth and warmer water with low salinity at the surface (Furman *et al.*, 2014).

In the beginning of the 20th century, the Baltic was an oligotrophic sea (Elmgren, 1989). Today the Baltic Sea is surrounded by nine countries accommodating ~85 million people (Leppäranta and Myrberg, 2009). Increasing population and intensified agriculture, wastewater discharge and nutrient loading initiated a eutrophication process in the 1950s, peaking around 1990 before countermeasures were initiated to reduce nitrogen (N) and phosphorus (P) levels (Reusch *et al.*, 2018).

Despite substantial reductions in nutrient loadings (Reusch *et al.*, 2018), eutrophication still poses a major problem that promotes toxic harmful algal blooms during summer. After the bloom, decay of the dead algae consumes oxygen, eventually creating bottom water redox conditions that liberate P from the sediments. This creates a seasonal self-sustaining “vicious cycle” (Elmgren and Larsson, 2001; Vahtera *et al.*, 2007; van Helmond *et al.*, 2020).

Over the past century, hypoxic areas in the Bornholm Basin (SD 25) and Gotland Deep (SD 28) have expanded from 5 000 km^2 to 60 000 km^2 (Carstensen *et al.*, 2014b). Today, the Baltic Sea contains the largest anthropogenic hypoxic area in the world (Carstensen and Conley, 2019) with widespread “dead zones” (Diaz and Rosenberg, 2008). The hypoxic state is continuously intensifying, exacerbated by both eutrophication and increased water temperature, the latter leading to reduced solubility of oxygen (Carstensen *et al.*, 2014a).

1.3 Baltic cod

Figure 2. Eastern Baltic cod in its natural habitat. Photo by Johan Candert, Deep Sea Productions.

Following the retreat of the most recent glaciation, the Littorina transgression around 8000 - 6000 years before present time (YBP) opened up the land barrier between Southwest Scandinavia and Central Europe for marine water to flood the freshwater Ancylus Lake, allowing for colonization of new marine fauna such as the top predator, Atlantic cod (Schmölcke, 2006). The brackish habitat is stressful for most aquatic organisms, as it exceeds the physiological tolerances of fully marine or freshwater species, creating conditions of low biodiversity (Elmgren, 1984; Hammer *et al.*, 1952). Although the salinity was higher in the Littorina Sea than in the Baltic Sea today (Westman *et al.*, 1999), Baltic cod (Fig. 2) nevertheless had to evolve and genetically adapt to estuarine salinities for osmoregulation and egg buoyancy (Berg *et al.*, 2015).

Cod spawn in batches, multiple times during the spawning season, ranging from April to August in the eastern Baltic Sea (Bagge *et al.*, 1994). Reproduction is restricted by both salinity and oxygen such that if the salinity is below the threshold for neutral egg buoyancy (14.5 ± 1.2 PSU) the eggs will sink to the bottom, becoming exposed to potentially lethal hypoxic or anoxic conditions (Nissling and Westin, 1997).

Today the anoxic areas overlap to great extent the historic spawning and feeding grounds for the cod population in the Eastern Baltic Sea, especially in the Gotland Deep and the Bornholm Basin (Cardinale and Svedäng, 2011). The commonly-used threshold for hypoxia (2 mg O_2/l) refers to the approximate oxygen level for fisheries collapse (Vaquer-Sunyer and Duarte, 2008) and with intensified hypoxia the "reproductive volume" suitable for spawning continues to decrease (Plikshs *et al.*, 1993). Hypoxia also compresses habitats, causing crowding and density-dependent processes with negative effects on cod condition (Casini *et al.*, 2021; Casini *et al.*, 2016; Limburg and Casini, 2018; 2019), metabolic performance, and hence less energy available for growth and foraging (Chabot and Claireaux, 2008; Claireaux and Chabot, 2016).

The International Council for the Exploration of the Sea (ICES) has divided the Baltic Sea into Sub-Divisions (SDs) for fishery management purposes (Fig. 1). Two cod stocks have been identified by genetic and tagging experiments, spatially positioning the Western Baltic cod stock in SD 22 - 24 and the Eastern Baltic stock in SD 25 - 32, with mixing occurring between the stocks in the Arkona Sea (SD 24) (Bagge et al., 1994).

The historic landings data are scarce, but indicate that although there were fluctuations in the cod spawning stock biomass it remained within the same range from the mid-1920s to the mid-1970s (Eero et al., 2008). The anthropogenic nutrient loading of the Baltic Sea and the increased primary production had an initial positive effect on cod recruitment from the 1940s to 1980s (Eero et al., 2011). The peak in cod spawning stock biomass in 1982-1983 was record high for the entire century as a response to reduced fishing pressure and unique favourable hydrographic conditions (MacKenzie et al., 2011) but followed by a major decline caused by a combination of overfishing and deteriorating environmental conditions (Casini *et al.*, 2009; Eero *et al.*, 2011).

Reduced maximum length, growth, condition, size at maturation and reproductive potential were all signs of a population in distress and in the 2010s the eastern Baltic cod population had plunged to such critically low levels that emergency measures led to a closing of the fishery in 2019 (ICES, 2019b; c).

Baltic cod, herring (*Clupea harengus membras*) and sprat (*Sprattus sprattus*) are three economically and ecologically key species controlling top-down processes of predator-prey interactions in the Baltic ecosystem (Rudstam et al., 1994). The collapse of Baltic cod initiated cascading effects in the food web resulting in a regime shift towards a bottom-up, planktivore dominated system, as massive predation on zooplankton by the increasing sprat population led to higher biomass of phytoplankton, further intensifying eutrophication. (Casini *et al.*, 2011; Casini *et al.*, 2008; Gårdmark *et al.*, 2015).

In the beginning of the 1990s, the spatial distribution of sprat moved towards the northeast Baltic Proper, while cod distribution shifted in the opposite direction (Casini et al., 2011). Signs of starvation emerged as prey availability decreased even further with reduced benthic fauna due to deterioration of deep habitats (Conley et al., 2009). In the 2000s, the Baltic seal population increased dramatically, targeting cod as prey and transmitting the parasitic nematodes cod worm (*Pseudoterranova decipiens*) and liver worm (*Contracaecum osculatum*), thereby increasing natural mortality (Mehrdana et al., 2014; Nadolna and Podolska, 2014).

1.4 Age estimation

Age information about fish is an important indicator of demographic, environmental and ecological changes and essential for stock assessments and management of fish populations. Knowledge of the age structure of a fish community allows for estimation of growth, maturation, longevity, reproduction, year class strength and mortality, all crucial biological parameters for achieving biologically and economically sustainable fisheries.

Growth zones form in several calcified parts and bones in teleost fish species. Scales, operculi, cleithra bones, vertebrae, fin rays, spines and otoliths are chronological structures used for age estimation (Casselman, 1983; Tzadik *et al.*, 2017). As early as 1759, the Swedish parson Hans Hederström studied the numbers of rings in vertebrae of pike (*Esox lucius*) and other fish species, coming to the conclusion that the number of growth zones was equivalent to the age (Hederström, 1759). Scales can be collected non-lethally, but unfortunately, scales stop forming growth zones when the fish no longer grows in size (Creaser, 1926). Scales can also be regenerated, resorbed during migration, or damaged by physical erosion (Kacem *et al.*, 2013). Otoliths form growth zones throughout the fish's entire life and are therefore considered the most reliable chronological structure (Campana, 1999). Moreover, otoliths are metabolically inert, meaning that the deposited material stays chemically stable and do not alter over time (Campana, 1999).

Otoliths consist mainly of aragonitic calcium carbonate crystals ($CaCO_3$), approximately 2% protein macromolecules forming an organic matrix, and a small fraction of incorporated trace elements (Asano and Mugiya, 1993; Baba et al., 1991; Campana, 1999; Hüssy et al., 2004). Teleost fishes have three pairs of otoliths, sagittae, lapilli and asterisci in the terminal organs of the endolymph-filled-semicircular canals within the inner ear (Popper *et al.*, 2005).

The otolith nucleus is formed by embryonal primordia (Lundberg et al., 2015) followed by a complex biomineralization process involving cellular and extracellular mechanisms (Allemand *et al.*, 2008; Hüssy *et al.*, 2020c). Otolith growth follows a circadian rhythm, continuously accreting material that forms daily, annual, and sometimes biannual increments (Morales-Nin, 2000; Pannella, 1971). Routine age reading of otoliths is based on the assumption that the strong seasonal contrasts of the growth zones are annually formed during the same time of the year. Regardless of spawning period, the 1st of January is assigned by convention as the arbitrary birth date for consequent ageing and quality assurance. Therefore, a fish hatched in spring and caught on December 31 the same year would belong to age class 0, but if it was captured one day later, the 1st of January it would be assigned to age class 1.

Imprecise age estimation can create major issues for fisheries management that depends on age-based stock assessment (Campana, 2001). This has proven to be the case for gadoid species such as the Atlantic hake (*Merluccius merluccius*) in the Bay of Biscay (De Pontual et al., 2006) and the eastern Baltic cod (*Gadus morhua*), both of which form irregular patterns of low contrasting growth zones in the otoliths (Berner, 1968; Eero et al., 2015; Hüssy, 2010). Baltic cod is traditionally aged by breaking the sagittal otolith at the sulcus acusticus to expose a transverse cross section through the nucleus (Fig. 3) and counting the number of translucent and opaque growth zones under a microscope with adjustable reflected light. (SLU-Aqua, 2012). The translucent zones are generally considered to periods of low growth during winter and the opaque zones to warmer growth periods during summer (Beckman and Wilson, 1995; SLU-Aqua, 2012). However, several studies have shown that the pattern can be reversed (Høie *et al.*, 2009) and related to temperature (Dannevig, 1956; Millner *et al.*, 2011; Neat *et al.*, 2008), or that opaque zones can form during growth in cold periods with favourable feeding conditions and translucent zones form during periods of starvation (Høie *et al.*, 2008).

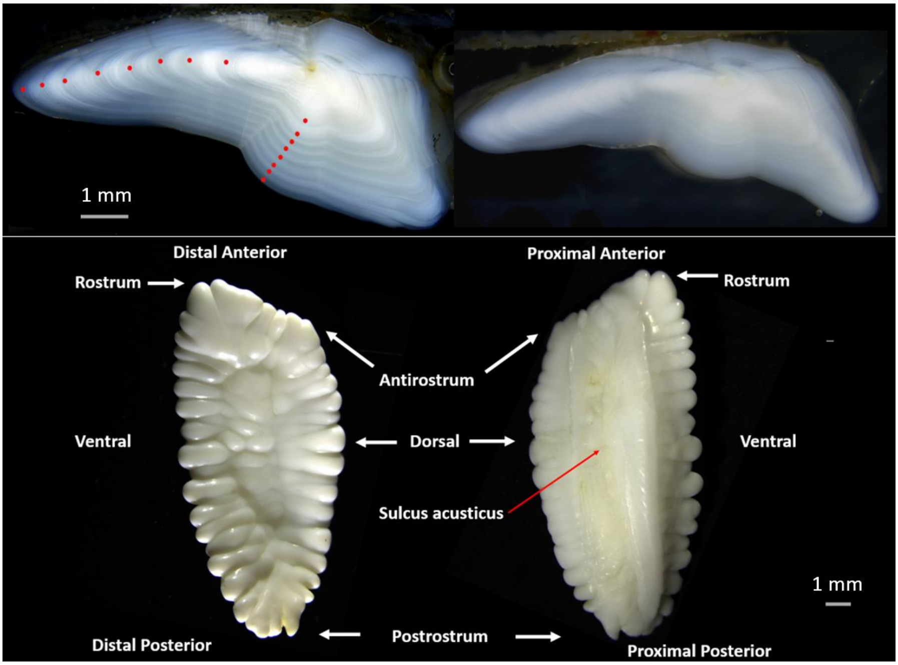

Figure 3. Top left: Transverse cross section of an eight-year-old eastern Baltic cod otolith with highly contrasting annual growth zones; red dots mark the translucent zones generally corresponding to cold periods with slow growth during winter. Top right: Baltic cod otolith with poorly contrasting growth zones and hence difficult to age. Bottom image: Morphology of a sagittal Baltic cod otolith. Photo montage by Yvette Heimbrand.

Experts in age determination of Baltic cod have been trying to resolve the ageing issue for over four decades by organizing international calibration workshops where participants have assigned ages to the same otoliths and discussed how to interpret the visible structures. However, false rings and irregular growth pattern caused consistently low precision (repeatability), accuracy (closeness between age readers' estimates and modal age) and percent agreement among age readers. An overview of the efforts made to solve the age determination issues is summarized in Hüssy *et al.* (2016b).

To examine the divergence in age readers' interpretation of annuli, and especially assigning the first annual ring, a calibration was set up in 2014 (ICES, 2014). Age readers marked annual structures on otolith images and the results revealed highly variable age estimates when validated against daily increment analyses. It was concluded that eastern Baltic cod otoliths do not follow the conventionally accepted growth pattern and that the traditional method did not meet the requirements of quality assured age data for stock assessment (ICES, 2014).

1.4.1 Age validation methods

Age validation is imperative for securing reliable biological parameters in analytical stock assessments for sustainable fisheries. There are other chemical age validation methods available, however, none suitable for the eastern Baltic cod. Long-lived fish species can be age validated by using radionuclides, relying on the decay rate over time (Andrews *et al.*, 2009; Campana *et al.*, 1990; Fenton *et al.*, 1991) or radiocarbon dating (Campana, 1997; Campana and Jones, 1998; Daugherty et al., 2020; Kalish et al., 1996; Kalish et al., 1997). Oxygen isotopes ($\delta^{18}O$) in marine organisms with calcified structures are considered temperature dependent when salinity is sufficiently constant; under these circumstances, tracking seasonal changes in water temperature can be used for age validation (Høie et al., 2004; Judd et al., 2018; Upton et al., 2012).

Baltic cod is however neither long-lived nor lives in constant salinity and cod of known-age raised in closed systems are not available. With the exception of tagging studies, alternative means of obtaining age validations on Baltic cod remain problematic.

An evaluation of three methods by Hüssy (2010) compared daily increment patterns, opacity profiles, and the traditional method. Opacity profiles did not consistently correspond to the width of the first year, validated with daily increments, and as this validation technique cannot be used for older ages, the need to identify an alternative method became an urgent matter. Tagging experiments by marking the fish with both an external tag and an internal tag, chemically marking the otolith as well as assessing seasonal patterns in otolith trace elemental uptake were methods suggested for validating Baltic cod age.

Chemical methods, especially those involving physiologically influenced trace elements in otoliths, were considered as possible candidates for developing a new age validation method for the eastern Baltic cod. This thesis addresses knowledge gaps and produces new knowledge for unlocking the otolith chemical chronology of age determination.

1.5 Otolith chemistry – trace elements and biomineralization

During a fish's lifetime it is exposed to the changing biotic and abiotic properties of the ambient water. Trace elements from the water can be transported through the gills or the intestines into the blood plasma and finally to the endolymph, available for incorporation into the otolith (Campana, 1999). The endolymph fluid also contains calcium and hydrogen carbonate ions for crystallisation of aragonite and organic macromolecules that form the proteinaceous matrix (Payan *et al.*, 2004). The function of proteins in otolith formation is increasingly studied (Thomas and Swearer, 2019) and recently Thomas *et al.* (2019) identified over 380 proteins in black bream (*Acanthopagrus butcheri*).

The organic matrix is divided into two categories, the "water soluble" fraction and the "insoluble" fraction, which represent respectively the dissolved content and remaining content post decalcification. The "water soluble" fraction mainly consists of non-collagenous proteins, glycoproteins, polysaccharides and lipoproteins (Asano and Mugiya, 1993; Baba *et al.*, 1991; Murayama *et al.*, 2000; Sasagawa and Mugiya, 1996; Thomas and Swearer, 2019) and the "insoluble" fraction, collagenous molecules (Borelli *et al.*, 2001; Davis *et al.*, 1995; Murayama *et al.*, 2004; Murayama *et al.*, 2002).

The opaque zones generally contain higher organic content than the translucent zones (Beckman and Wilson, 1995). Campana (1999) suggested that trace elements could substitute for calcium, be trapped in the interstitial space between crystals, or bind to the organic matrix and thereby be incorporated into the otolith.

The biomineralization process is complex, linked to different drivers and still not fully understood. Nevertheless, otolith chemistry has gained increasing interest as an application in fisheries science based on the concept that trace elemental incorporation is a continual process as otoliths grow, and that otolith chemical composition reflects some combination of environmental and physiological changes experienced by a fish over its lifetime (Begg et al., 2005). These properties enable a wide variety of applications including stock identification, migration behaviour, reconstruction of water temperature and salinity, climate cycles, chemical mass marking and age validation (Campana, 1999).

Here follows a brief presentation of trace elements assessed for seasonal patterns. New insights found within the framework of this thesis as well as results from other recent studies of chemical age estimation of Baltic cod will be presented in the Results and Discussion.

1.5.1 Strontium

H.T. Odum's doctoral thesis, "The Biogeochemistry of Strontium", published in 1950 was one of the first studies of otolith chemistry, stating that the otolith strontium to calcium (Sr:Ca) ratio was highly correlated with the corresponding ratio and salinity of the surrounding water (Limburg, 2004; Odum, 1950).

Dissolved in water, this alkaline earth metal forms Sr^{2+} with the same ionic charge as Ca^{2+}. Given similar ionic radii, Sr^{2+} readily substitutes for Ca^{2+} in the aragonitic matrix (Bath *et al.*, 2000; Campana, 1999; Doubleday *et al.*, 2014; Kraus and Secor, 2004). Several studies have focused on strontium and found the ratio of Sr relative to Ca in the water has a strong, positive linear relationship in otolith uptake (Chowdhury and Blust, 2001; Elsdon and Gillanders, 2002; Kraus and Secor, 2004).

The Sr:Ca level in water increases with higher salinity in many brackish systems (Surge and Lohmann, 2002) providing the precondition for studies of diadromous fish species migration routes between marine and freshwater (Walther and Limburg, 2012) as well as provenance (Lill *et al.*, 2018). However, Sr in freshwater varies considerably; in regions with Sr-rich geology and soils it may even exceed marine concentrations (Kraus and Secor, 2004). The otolith strontium –salinity calibration curve shows highest sensitivity and a sharp increase at < 10 PSU, levelling out at higher salinities (Hemmer-Hansen *et al.*, 2020; Walther and Limburg, 2012). Therefore it is possible to detect migration patterns within low salinity, estuarine habitats (Chowdhury and Blust, 2001; Gillanders, 2005).

Temperature may also influence strontium uptake in otoliths; for example, in a study of laboratory-reared larval cod, Sr:Ca increased as temperature declined from 16 to 3°C (Townsend et al., 1995), and the same trend was supported by Stanley *et al.* (2015) performing similar studies on juvenile cod. The Baltic Sea exhibits a temperature and salinity gradient distributed both vertically and horizontally from north to south (Furman et al., 2014) resulting in complex migration patterns reflected by Sr:Ca in Baltic cod otoliths as well as ontogenetic habitat shifts (Limburg *et al.*, 2011).

1.5.2 Barium

The barium (Ba) content in otoliths reflects the abundance of this alkaline earth element in the surrounding water (Bath *et al.*, 2000; Elsdon and Gillanders, 2003b; Miller, 2009; Milton and Chenery, 2001). Biogeochemical cycles in the sea as well as riverine input influence the Ba content in marine water. Depending on the freshwater catchment rock type, weathering and flow rate of suspended particles, Ba dissolves where freshwater mixes with marine water under low salinity estuarine conditions (Coffey *et al.*, 1997).

Laboratory experiments have indicated fastest desorption of Ba in 1–2 PSU (Coffey, 1994). In higher salinities dissolved Ba precipitates as barite ($BaSO_4$) but under euxenic conditions Ba can re-dissolve again, resulting in the highest concentration at depth towards depletion at the surface (Dehairs *et al.*, 1980).

The effect of temperature and salinity and the combination of the two factors have been tested in several laboratory experiments with varying results. In constant high salinity (20 PSU) there was no effect of temperature when comparing otolith Ba:Ca of spot (*Leiostomus xanthurus*) in 20°C and 25°C (Bath *et al.*, 2000). Juveniles of the same species, studied in two treatments of salinity (15 and 25 PSU) and four temperatures (17, 20, 23, and 26°C) only showed a salinity effect but no effect of temperature (Martin and Thorrold, 2005). Juveniles of another species, the southern black bream (*Acanthopagrus butcheri*) showed increasing otolith Ba:Ca concentration with increasing water temperature (16 - 20°C) in 5, 17, and 30 PSU (Elsdon and Gillanders, 2002). Nelson *et al.* (2018), studying Gulf killifish (*Fundulus grandis*), identified opposing influences of salinity and temperature.

Gulf killifish had highest otolith Ba:Ca in low salinities and high temperatures, showing an exponential decrease in otolith Ba:Ca with salinity as well as exponential increase with temperature. Field experiments studying the effect of salinity on otolith Ba incorporation have shown increasing otolith Ba:Ca concentration with decreasing salinity (Milton and Chenery, 2001; Mohan et al., 2012). As the Ba otolith uptake follows the Ba gradient in brackish waters, with highest concentrations near coastal riverine inputs, it can be utilized as a chemical discriminator between habitats (Mohan et al., 2012; Secor et al., 2001; Tabouret et al., 2010), among populations (Avigliano *et al.*, 2015) or for studies of natal homing behavior (Thorrold *et al.*, 2001) or migration pattern (Walther and Limburg, 2012).

In the North Pacific Ocean, seasonal coastal upwelling events during summer deliver Ba-rich water to the surface resulting in seasonal Ba:Ca signatures in otoliths of eulachon (*Thaleichthys pacificus*) suggested as markers of annual growth zones by Benson et al. (2019). However, this seasonality is environmentally linked to regional oceanographic and geological properties which might vary considerably (Bath et al., 2000; Miller, 2011; Morales-Nin et al., 2005) and there is scant evidence for Ba being under physiological control, reflecting growth (Sturrock et al., 2014). For Baltic cod, an ontogenetic inverse relationship of Ba in relation to Sr suggests a coastal, low salinity juvenile nursery habitat.(Limburg *et al.*, 2011).

1.5.3 Manganese

Manganese (Mn) is geologically widely distributed in minerals, but also can form large nodules on the sea floor (Mero, 1962). Dynamic redox conditions between oxic and euxinic zones, formed in combination with raised levels of hydrogen sulfide (H_2S), regulate the Mn concentration in the water column, provided that Mn is present in the sediment (Bauer, 2014; Reddy and DeLaune, 2008; Thamdrup, 2000). In the Baltic Proper, redox zones are found both in the water column and in the sediment due to stratification, reducing manganese to Mn^{2+} and Mn^{3+} in dissolved form (Bauer, 2014), with concentrations generally increasing with depth (Lenz *et al.*, 2015).

In otoliths, Mn has been found both protein-bound in the organic matrix (Izzo et al., 2016; Thomas and Swearer, 2019) and unbound, suggested to replace Ca in aragonite due to similar ionic radius (Thomas et al., 2017). In cod otoliths however, Miller *et al.* (2006) did not detect Mn in the protein fraction, indicating absence or content below detection limits.

Studies on the effect of salinity and temperature on otolith Mn:Ca have reported inconsistent results (Elsdon and Gillanders, 2002; Martin and Wuenschel, 2006; Miller, 2009; Stanley *et al.*, 2015) and also laboratory and field studies of the otolith Mn:Ca response to water Mn:Ca concentration have given contradictory results.

Laboratory trials of black bream (*Acanthopagrus butcheri*) (Elsdon and Gillanders, 2003a) and juvenile black rockfish (*Sebastes melanops*) (Miller, 2009) found no relationship, while field experiments studying striped bass (*Morone saxatilis*) (Mohan et al., 2012) and juvenile Atlantic croaker (*Micropogonias undulatus*) (Thorrold and Shuttleworth, 2000) saw a positive relationship with water concentrations. Limburg *et al.* (2011) found otolith Mn:Ca in cod to be linked to the extent of hypoxic areas in the Baltic Sea.

By assessing the dynamic variation over time, otolith Mn:Ca can track historic exposure to hypoxia (Altenritter *et al.*, 2018; Limburg and Casini, 2018; Limburg *et al.*, 2015; Mohan and Walther, 2016). High Mn:Ca peaks in the otolith core have been attributed to maternal transfer of Mn, affecting the embryonic formation of the primordium (Brophy *et al.*, 2004; Ruttenberg *et al.*, 2005) and used as a natural chemical tag for stock discrimination (Artetxe-Arrate *et al.*, 2019).

Limburg et al. (2015) found that apart from demonstrating Mn:Ca to be a useful proxy for hypoxia across taxa, otolith Mn:Ca was also affected by growth, showing highest response within the first two annual growth zones thereafter declining with age. In a normoxic laboratory experiment on juvenile Pacific cod (*Gadus macrocephalus*), otolith Mn:Ca was positively associated with growth (Miller and Hurst, 2020), Hence, interpretation of otolith Mn:Ca can be complex, as it is associated with both exogenous and endogenous factors with the implication that high otolith Mn:Ca can indicate a negative effect on growth due to hypoxic exposure or a positive effect correlated to ontogeny.

1.5.4 Magnesium

The magnesium (Mg) concentration in the sea (5 x 10^{-2} M) is higher than in freshwater (< 10^{-4} M), (Frausto da Silva and Williams, 1991). Several studies have used Mg:Ca in calcite from foraminifera and ostracod shells as a seawater paleotemperature proxy, the methodology is reviewed by Lowenstein and Hönisch (2012) and based on that Mg in calcite precipitated from seawater is positively correlated with temperature. Otolith Mg:Ca, however, does not appear to accurately reflect the ambient water composition (Dorval et al., 2007; Hamer and Jenkins, 2007; Walsh and Gillanders, 2018; Woodcock et al., 2012)

Studies have reported conflicting results for the relation between otolith Mg:Ca and environmental factors, such as temperature (Miller and Hurst, 2020), salinity (Dorval et al., 2007), or both (Elsdon and Gillanders, 2002; Martin and Thorrold, 2005; Wells et al., 2021). Woodcock et al. (2012) did not find any clear link between diet and otolith Mg:Ca in silver perch (*Bidyanus bidyanus*) and neither did Marohn et al. (2009) in European eel (*Anguilla anguilla*). Instead, otolith Mg:Ca seems to be physiologically regulated, driven by metabolism (Grammer et al., 2017; Limburg et al., 2018) and correlated with somatic growth (Miller and Hurst, 2020; Stanley et al., 2015; Sturrock et al., 2015). Hamer and Jenkins (2007) discovered otolith Mg:Ca to be species specific and positively correlated with otolith accretion rate, with consistently higher levels in snapper (*Pagrus auratus*) than sand flathead (*Platycephalus bassensis*).

Thomas et al. (2017) found Mg only in the fraction that is not bound to protein in the endolymph. Mg has a smaller ionic radius than Ca, unlikely to compete with Ca in the aragonite, however when hydrated the radius is larger. While information about Mg otolith uptake is scarce, the limited utility of Mg as an environmental indicator, suggests that incorporation mechanisms are likely more associated with random trapping or proteins in the organic matrix.

Hüssy *et al.* (2016a) compared maxima and minima in concentration in trace elemental profiles with opacity patterns in otoliths from North Sea cod and western Baltic cod, easy to age, as well as eastern Baltic cod, difficult to age. Variations in otolith Mg:Ca corresponded to opacity profiles and growth, with higher ratios in summer and lower in winter. This periodicity makes Mg:Ca potentially suitable for chemical aging in seasonally varying environments.

1.5.5 Phosphorus

Phosphorus (P) is one of the most widely studied nutrients in the world and a key element for the evolution of life on earth. P is contained in every living cell and therefore vital for plants and animals. It plays a fundamental role in cellular metabolism for energy storage and is an essential part of the cell membrane and genetic material. In freshwater and estuarine aquatic ecosystems, P is usually the most important limiting nutrient involved in the eutrophication process (Correll, 1999; Correll, 1998; McCrackin *et al.*, 2018; Schindler, 1977; Schindler *et al.*, 2008).

Under reducing conditions, marine sediments can act as a source for phosphorus. Conley et al. (2002) found that dissolved inorganic phosphate was positively correlated to the extent of hypoxic bottom areas in the Baltic Sea, but not to the total P nutrient load, indicating significant legacy effects of past eutrophication on current biogeochemical cycling of P.

There are few otolith studies involving P, most likely due to the limited utility in environmental studies. Otolith uptake of P shows no response to ambient water concentrations and is regarded as physiologically regulated (Campana et al., 2000; Campana, 1999). In a study of kutum (*Rutilus frisii*) in the Caspian Sea, otolith P:Ca did not reflect migration between habitats (Bani et al., 2020).

Elevated levels of P:Ca in otolith cores have been reported (Shima and Swearer, 2009) and associated with phosphorylation of proteins or other macromolecules during the nucleation process and subsequent increment formation with P incorporated in the organic matrix (Thomas et al., 2017). Otolith core P:Ca composition in combination with Mg, Sr, Ba and boron (B) contributed to identify provenance of broadbill swordfish (*Xiphias gladius)* in the Indian Ocean (Darnaude et al., 2020), but the reason behind habitat differences in concentration remain unknown.

For quantification of physiologically driven incorporation coupled to otolith growth, seasonal variation in P:Ca has been assessed for chemical age estimation which will be elaborated further in the thesis.

1.5.6 Copper and Zinc

In aquatic environments, levels of the transition metals copper (Cu) and zinc (Zn) are generally low in the water column and are instead bound to dissolved organic matter and sediments at the bottom. Cu and Zn are essential trace nutrients for fish (Watanabe *et al.*, 1997) but can be toxic at high levels and derived from both water and diet (Handy, 1996; Wood, 2001).

Milton *et al.* (2000) explored the use of otolith Cu and Zn as indicators of polluted water, contaminated with heavy metals but did not find any response to water chemistry in otoliths from barramundi (*Lates calcarifer*). Similarly, otolith Zn:Ca in larval and juvenile Atlantic croaker did not track coastal pollution (Thorrold *et al.*, 1997). However, Arslan and Secor (2005) reported that high exposure to Cu and Zn may increase incorporation rates as elevated otolith Cu:Ca and Zn:Ca in American eels (*Anguilla rostrata*) were directly associated with enriched contaminated water concentrations. The contradicting results suggest that environmental influence may only result from extreme exposure to contaminated water (Barbee *et al.*, 2013; Miller *et al.*, 2006; Milton and Chenery, 2001).

Jackman *et al.* (2016) detected significant chemical asymmetry in otoliths from the flatfish winter flounder (*Pseudopleuronectes americanus*) with higher Zn:Ca and Cu:Ca in the blind-side otolith, hypothesizing that the orientation towards the sediment enabled higher absorption of transitional elements caused by redox reactions.

By dissolving whole otoliths from cod, Miller *et al.* (2006) found 70% - 100% of Cu and 40% - 60% of Zn in the soluble part of the protein matrix, suggesting that fish physiology regulates uptake and incorporation. High concentrations of Zn:Ca and Cu:Ca in the otolith core with subsequent decline with age has been reported for several species, such as cod (Hüssy *et al.*, 2016a), mackerel (*Scomber japonicus colias*) (Papadopoulou *et al.*, 1978) and pink snapper (*Pagrus auratus*) (Ranaldi and Gagnon, 2010), indicating ontogenetic incorporation. In European plaice (*Pleuronectes platessa*) otolith Zn:Ca in females decreased during spawning and was negatively correlated to the gonadosomatic index of females post spawning (Sturrock *et al.*, 2015). The same study also reported seasonal variations in Zn:Ca, positively correlated to temperature and the opposite in Cu:Ca for both temperature and condition.

Species specific differences in Zn:Ca concentration have been reported with higher levels in otoliths from species within the family of Salmonidae than other taxa (Lill *et al.*, 2014; Limburg and Elfman, 2010). In several species belonging to the Salmonidae, Esocidae and Gadidae families, visual growth zones have displayed a synchronous pattern with Zn:Ca, where minima in concentration corresponded to annual translucent zones (Friedrich and Halden, 2010; Halden *et al.*, 2000; Limburg and Elfman, 2010). In the Bering Sea, Benson *et al.* (2019) used Zn:Ca transects to identify the first winter zone in eulachon otoliths, which was difficult to detect with visual methods. The results from these studies suggested that seasonal otolith Zn:Ca patterns are related to fish growth. As a result, (Hüssy *et al.*, 2016a) included Zn but also Cu for identifying potential elements for chemical aging of cod. The seasonal patterns in chemical profiles of both Cu and Zn correlated with opacity profiles of visual growth zones in cod otoliths from the North Sea, western- and eastern Baltic Sea suggesting a common incorporation mechanism and candidates for further evaluation of the development of a new chemical age estimation method.

2. Aims of thesis

The overall aim of this thesis was to provide a long-term solution for age estimation and validation by developing a new method based on otolith chemical proxies displaying seasonal variations in elemental concentration. As a secondary aim, I tested the new method on otoliths from eastern Baltic cod, a threatened stock, known for being difficult to age due to low contrast, often confusing annual growth zones causing major challenges in analytical assessments for fisheries management. To reach these aims, I used both empirical and theoretical approaches.

In **Paper I**, several fish species of different taxa were examined to assess if magnesium, a physiologically regulated trace element incorporated into otoliths reflected seasonality, i.e. age. A hypothesis was developed that otolith magnesium biomineralization is regulated by metabolism.

In **Paper 2,** a new, chemical age estimation method for Baltic cod was developed, based on otolith chemistry. The method was tested in an age calibration exercise where experienced age readers practising the traditional, visual method on otolith images, estimated age on the same set of cod otoliths from the Baltic and North Sea as untrained readers using the new chemical method.

In **Paper 3,** I evaluated the chemical age estimation method by assessing otolith chemical response in magnesium and phosphorus in relation to growth and used daily increments for validation of seasonal patterns.

In **Paper 4,** the new chemical method was used to interpret life history transects at the annual scale, combining age with chemical proxies of the marine environment. I used a rare collection of Neolithic otoliths to establish baselines of hypoxia, metabolic status, and growth. Baltic cod otoliths from 1927-2018 were then analysed to reconstruct a near-century of environmental and physiological change.

My research follows the conceptual model (Fig. 4) and will fill crucial knowledge gaps for improving the management of the eastern Baltic cod. The new chemical method will serve as an a useful tool for reconstructing time-resolved life histories of individual fish and guide in development of chemical age estimation methods of other difficult to age species.

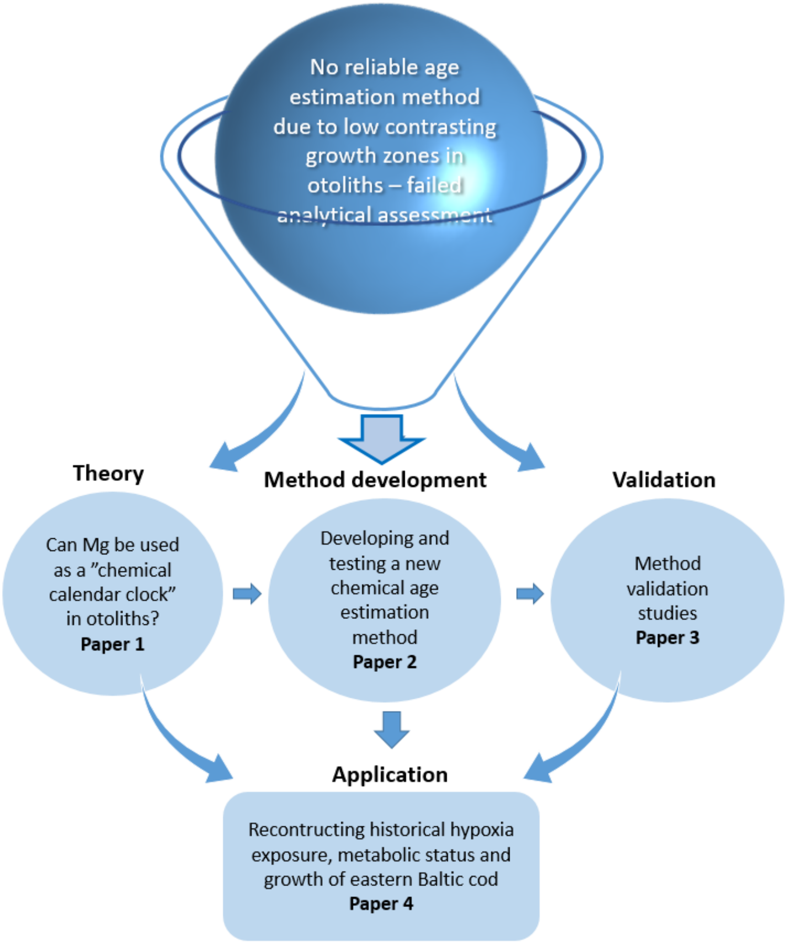

Figure 4. Conceptual model of the research approach of the thesis.

3. 3. Materials and Methods

3.1 Sample collection

Paper I surveyed a range of species across the phylogenetic spectrum and included several species; European eel (*Anguilla anguilla*), Atlantic herring (*Clupea harengus var. membras*), Atlantic cod (*Gadus morhua)* and European flounder *(Platichthys flesus)* from the Baltic Sea, American eel (*A. rostrata*), American shad (*Alosa sapidissima*) and blueback herring (*A. aestivalis*) from the Hudson River NY, USA and shrimp flounder *Gastropsetta frontalis*), Greenland halibut (*Reinhardtius hippoglossoides*) and American plaice (*Hippoglossus platessoides*) from the western Atlantic, collected from fishery independent surveys by NOAA's Northeast Fisheries Science Center in Woods Hole, Massachusetts, USA.

The Baltic Sea cod otoliths in **Paper II** and **IV** came from archived material and included samples representing 1927 - 2018 and the Neolithic period ~4500 years before present time, as well as subsets of cod otoliths from the North Sea (Fig. 5).

Figure 5. Eastern Baltic cod otoliths from the Neolithic time period (brown) and modern time (white). Manganese (shown here in its solid form) was used as an otolith chemical proxy for hypoxia. Photo by Karin Limburg.

The samples from the Baltic Sea in **Paper I, II** and **IV** were collected from fishery independent trawl surveys carried out by the former Swedish Board of Fisheries (currently the Swedish University of Agricultural Sciences, Department of Aquatic Resources) as well as from commercial landings. In **Paper III**, Baltic cod otoliths were received from field experiments and fishery independent trawl surveys conducted by SLU and from the Baltic Sea Science Centre at Skansen Museum, Stockholm Sweden.

3.2 Environmental data

Environmental data were compared to otolith chemical proxies for hypoxia and metabolic status in **Paper IV** of hypoxic (< 2 ml/l O_2) and anoxic (< 0 ml/l O_2) volumes (km^3) in the Baltic Sea per year, retrieved from Hansson *et al.* (2019). Long-term records of dissolved oxygen (DO, mg/L), temperature (°C), and salinity (PSU) measured at the monitoring station BY15 in the Gotland Deep were extracted from the Nest system in the Baltic Nest Institute's Baltic Environmental Database (BED), http://nest.su.se and included data from SMHI (the Swedish Meteorological and Hydrological Institute, https://sharkweb.smhi.se/) and other sources. These were used to calculate DO as percent saturation (Garcia and Gordon, 1992), a more physiologically relevant metric than DO concentration per se. Temperature, salinity, and both expressions of DO were correlated against cod hypoxia exposure and metabolic status.

3.3 Aquarium and field experiments

Eastern Baltic cod otoliths included in **Paper II** were received from two sets of samples, and utilized for assessing the relationship between otolith chemistry and growth.

3.3.1 Aquarium experiment

I received Eastern Baltic cod otoliths in two batches (2019 and 2020) from the Baltic Sea Science Center, a public aquarium at Skansen museum in Stockholm, Sweden. The cod were not individually tagged and time in captivity unknown. All had been euthanized due to an eye infection and saved for study. I used the otoliths to examine how otolith chemistry was affected by the transfer from ambient conditions in the southern Baltic Sea (SD 24 and 25) to Skansen. The aquarium maintained a constant salinity (16-17 PSU) and temperature (12.5 – 13.5 °C) year round in normoxic water and abundant food supply, providing good conditions for growth. The otolith growth reflecting time in the aquarium was visible and could be measured from otolith images (Fig. 6). The corresponding growth estimated with otolith chemistry was measured from chemical profiles, clearly showing when the fish entered the aquarium.

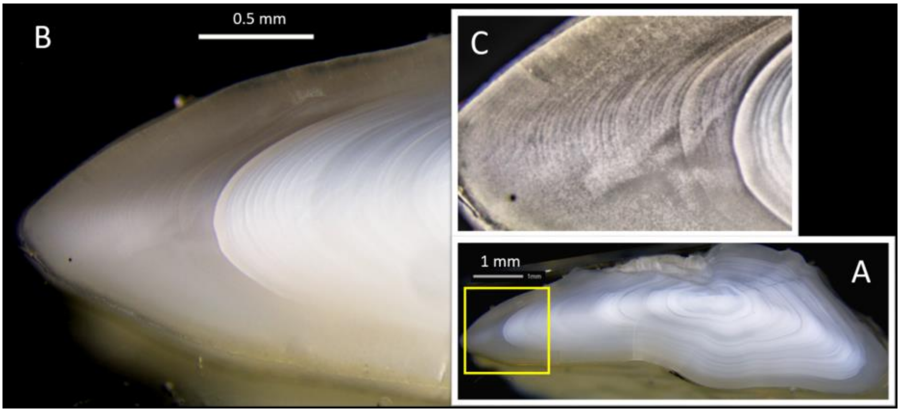

Figure 6. Baltic cod otolith from the aquarium experiment illustrating fast otolith growth accreted during the time in the aquarium in a whole transverse cross section (A), dorsal edge in detail (B) as well as broad growth increments within (C). Reproduced from Paper III.

3.3.2 Field experiment

A feeding experiment on eastern Baltic cod was conducted in the autumn of 2018 and during spring in 2019. The cod were maintained in sea pens along the coast outside Karlshamn in the southern Baltic Sea (SD 25) and fed with pieces of locally caught herring. The last 100 µm on the dorsal otolith edge was estimated to reflect growth during the feeding experiment. The value of the chemical ratio 100 µm from the edge was subtracted from the value at the edge. The difference reflected the chemical response. The cod were externally tagged and I used multiple regression analyses to assess the response in otolith Mg:Ca and P:Ca as functions of both internal and external factors. These included: pre- and post-experiment in length (ΔL), weight (ΔW) and condition measured as Fulton’s K (ΔK). Additional variables included hepatosomatic index (HSI), gonadosomatic index (GSI) cumulative daily mean temperature (CumT) experienced by each fish, feeding amount per kg biomass starting weight per pen, gender, year and the liver worm (*Contracaecum osculatum*) infection intensity (LWI), measured as the number of parasites per gram liver at the end of the trial. Finally, I used lifetime cumulative otolith Mn:Ca (Cum.Mn) as a measure for pre-experimental exposure to hypoxia.

3.4 Otolith preparation method for age estimation and chemistry

The growth histories in otoliths were exposed by assessing visual and chemical patterns in transverse cross sections. For preparation, sagittal otoliths were embedded in epoxy (Struers EpoFix) and then sectioned with a diamond saw. Each section was polished by hand with grinding paper from P400 down to a grit size P2500 until the otolith core became visible and finally polished with Struers diamond suspension 0.5 µm and cleaned with ethanol. The transverse cross sections were photographed with a microscope camera and placed on petrographic glass slides with double-stick tape.

For assessing patterns of daily increments in **Paper III**, I polished the otoliths to a thickness of approximately 100-300 µm. The otolith microstructure was viewed with a Leica compound microscope at 40X magnification and projected with a video camera. Distances between annual growth zones were measured with the Leica's LAS X microscope control software (leica-microsystems.com).

3.5 Age estimation

Traditionally, age reading of cod otoliths is done by counting visible growth zones in the cross-section created when the otolith is broken in half at the nucleus, based on the assumption of consistent formation of seasonal contrasting opaque and translucent growth zones, corresponding to summer and winter, respectively (ICES, 2014).

In **Paper II**, I developed a new chemical age estimation method based on interpreting seasonal patterns in otolith chemical profiles reflecting individual fishes' entire life transect, from the core (birth) to the edge (death) of different trace elements in ratio to calcium. The sample collection consisted of cod otoliths from the last four decades, caught in the Baltic and the North Sea. Without any documented manual of chemical interpretation, the new method was tested for the first time and compared to the traditional, visual method conducted by highly experienced age readers using otolith images in an age calibration exercise. The experiment was carried out on a relatively new platform called SmartDots (ICES, 2018) which was designed for the purpose of comparative aging exercises such as this.

Age readers using the traditional method placed marks on otolith images on the structures they interpreted as annual growth zones along a predefined line from the otolith core to the dorsal edge following the maximum growth axis. Readers using the chemical method annotated annuli based on seasonal patterns in concentration maxima and minima along the chemical profiles of different trace elemental ratios.

All readers, regardless of method marked their interpreted annuli based on visual structures or chemistry on an otolith image for direct comparison of visual structures, opacity level and otolith chemistry. The age readers also assigned a ranking score for otolith image readability to examine if they perceived otoliths from a certain area or time period to be more difficult to interpret. In a similar way, different chemical ratios were ranked to assess reader's confidence in identifying chemical annuli.

3.6 Otolith chemical analyses

Analytical advances in geochemistry and paleoclimate research have been adopted by fisheries researchers, enabling more development and discovery that links the otolith chemistry to biology and ecology. To quantify and visualize the trace elemental composition in otoliths is an important tool for providing spatial and time-resolved information about the life history of individual fish and otolith chemistry is becoming a standard tool in fisheries research (Walther et al., 2017). Trace elemental and isotopic concentrations can be detected at levels as low as parts per billion (ppb) and imaging techniques can provide line transects and two-dimensional imaging of elemental maps (Fig. 7) for comparison between chemical patterns and otolith structures, correlated with events in the fish's life (Becker *et al.*, 2014; Limburg and Elfman, 2017).

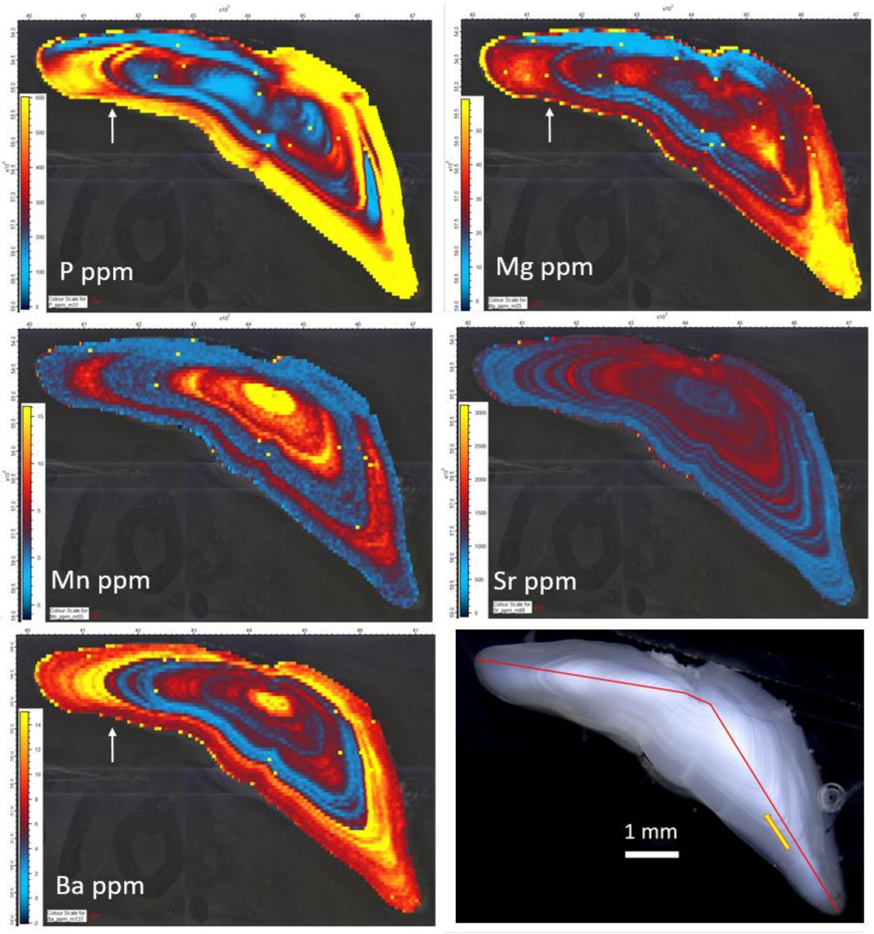

Figure 7. Two dimensional trace elemental maps of a transverse cross section of an eastern Baltic cod otolith received from the aquarium at Skansen. Distinct increases in P, Ba and Mg concentration indicate the transfer from the sea to the aquarium (white arrows). Photo montage by Yvette Heimbrand.

3.6.1 Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)

Currently, no single instrument can measure the entire range of elements and isotopes in otoliths. To retrieve the most chemical information of the otolith elemental composition for the purpose of this thesis, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was selected.

Trace elements reflecting:

- Environment: strontium (^{88}Sr) and barium (^{137,138}Ba)
- Environment and physiology: manganese (^{55}Mn)
- Physiology: magnesium ($^{24, 25, 26}$Mg), phosphorus (^{31}P), copper (^{63}Cu) and zinc (^{64,66}Zn)

were quantified as ppm and often reported in ratio to calcium (measured as ^{43}Ca) at two facilities, SUNY-ESF, Syracuse, NY, USA and at Lund University in Lund, Sweden (Fig. 8).

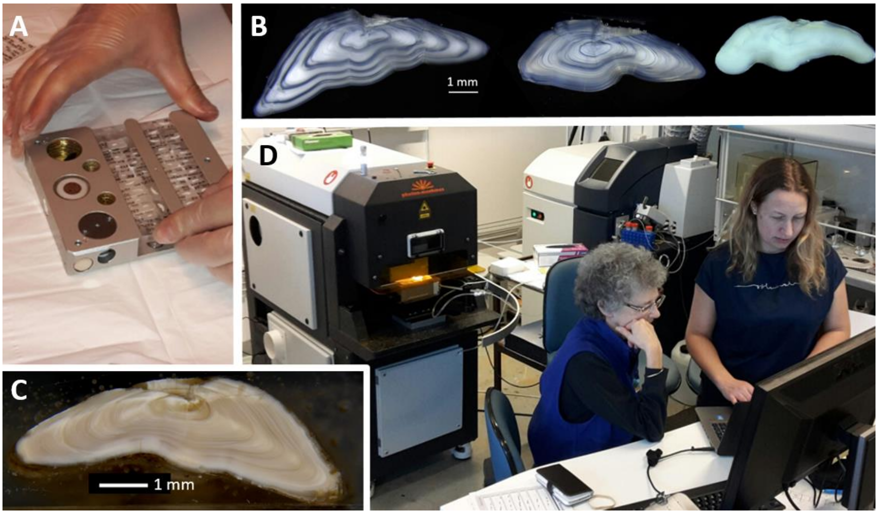

Figure 8. A: sample holder, B: transverse cross sections of otoliths from a North Sea cod (left), and two eastern Baltic cod (middle and right), C: eastern Baltic cod from the Neolithic time period, D: Yvette Heimbrand and Karin Limburg analyse otoliths at the LA-ICP-MS facility at Lund University, Sweden. Photo by Martina Blass SLU.

3.6.2 Interferences

It is important to account for possible interferences of the analysed trace elements and adjust the instrument operating conditions accordingly. Divalent ion interferences may appear when sample ions with twice the mass are equal to the mass/charge ratio of the analyte (Thomas, 2013). For example, Thorrold and Shuttleworth (2000) discovered that $^{48}Ca^{2+}$ was the source of constant divalent ion interferences for ^{24}Mg. This was the case for the analyses at the facility at ESF, and was discovered when we analysed the same set of otoliths in a comparison for quality assurance at both facilities The interference was constant and adjusted multiplying by a factor of 0.6 and all Mg concentrations obtained at ESF consequently adjusted for in the data used in this thesis.

3.7 Statistical approaches

Statistical analyses in this thesis were done with R version 3.5.2 (R Core Team, 2019) and Excel. For data reduction and processing of the otolith chemical analyses included in **Paper I-IV**, I used the software IOLITE in combination with Igor Pro to achieve line transects and two-dimensional images. (Hellstrom *et al.*, 2008; Paton *et al.*, 2011). This program enables data reduction and the creation of two- and three-dimensional images, color-coded for each element, or isotope, representing abundance or concentration levels for single-matrix samples using parallel adjoining line scans (Ulrich *et al.*, 2009). **In Paper III**, we followed the method of Hüssy *et al.* (2020b), were peaks and valleys in trace elemental concentration were identified along the chemical profiles with a loess smoothing algorithm and the distance from the core to local minima recorded. I examined the concordance of chemical and daily increment patterns with a linear mixed effects model (LME), using chemical minima as the response variable, the number of annual growth zones identified with daily increments as a fixed effect and individual fish as a random effect. In **Paper IV**, I used the new ageing method to parse chemical annulus per calendar year based on year of catch. That enabled comparisons on an annual level in a longterm perspective and comparison of different age classes. Several repeated measurements were made on each otolith depending on age, therefore a mixed effect model taking into account random effects of individual fish was applied for predicting average length at age for different time periods.

4. Results and Discussion

In this thesis, seasonal patterns in fish otolith magnesium (Mg) were hypothesized to correspond to annual growth zones and possible to use as a “chemical calendar clock” for age estimation (**Paper I**). A new method was developed, based on otolith chemistry and compared to the traditional, visual method in an age calibration exercise (**Paper II**). The new method was thereafter tested and validated (**Paper III**). Eastern Baltic cod otoliths from the Neolithic time period were chemically analysed and age estimated with the new method to establish baselines of hypoxia, metabolic status, and growth (**Paper IV**). Finally, the new method was used to reconstruct a near-century of environmental and physiological change recorded in eastern Baltic cod otoliths from 1927-2018 (**Paper IV**).

4.1 Theory – Metabolically regulated trace elemental uptake

In **Paper I**, otoliths were examined from ten species across five families spanning from less phylogenetically derived (Anguillidae) to highly derived (Pleuronectidae) for evidence of Mg:Ca responding to metabolic and/or growth. Samples were chosen to contrast lifestyles (e.g., cold stenothermal Greenland halibut vs. warm eurythermal American plaice and Baltic flounder), food availability vs. temperature (American shad), extreme metabolism in early life (larval blueback herring, metamorphosing glass eels), and seasonality (Baltic flounder, herring, and cod).

This varied approach was used to build an empirical case for otolith magnesium incorporation to be regulated by physiology. A two-step mechanism was thus proposed: that the magnesium ion (Mg^{2+}), although smaller than Ca^{2+}, readily becomes doubly hydrated, which then increases its ionic radius to approximately 11 times that of hydrated Ca^{2+}. As such, hydrated magnesium requires more energy to transport it into the otolith saccular chamber. The proximal ion chambers are surrounded by a dense meshwork of mitochondria, literally serving as "powerhouses" to regulate ion transport, and it was proposed that these are active when metabolism is generally high, such as during summer and other optimal growth conditions. Further, once inside the chamber, incorporation into the otolith matrix was proposed to be limited by the relative abundance of soluble binding proteins. This paper (Limburg *et al.*, 2018), putting forth hypotheses, was intended to raise interest in the community to explore this and other incorporation mechanisms further.

Applicability- Otolith chemistry as an age estimation method

Based on the theoretical framework of **Paper I,** a new chemical age estimation method was developed. The method was tested in an age calibration exercise, where the same set of cod otoliths were age determined by experienced readers using the traditional method of counting visible annual growth zones and untrained readers interpreting seasonal patterns in trace elements (**Paper II).**

The new chemical method gave higher percent agreement 74.2% (Baltic Sea) and 73.3% (North Sea) among readers compared to the visual method, 50.2% and 54.2% respectively. The precision error (average coefficient of variation) was more than double and the average relative bias threefold higher for the visual method. In addition, the average age estimates for the visual method were significantly higher than for the chemical method.

The acceptance criterion for percent agreement among age readers is generally set to 80% for most species, (Eltink, 2000; ICES, 2019a), but depending on age, area and how difficult the fish species is to age, it can be lower or higher. For example, the acceptable percent agreement for the difficult-to-age European eel (Anguilla anguilla) is 70% (SLU-Aqua, 2012). The fact that the chemical method exceeded this level in the first trial was promising for potentially providing a solution for accurate age estimates of eastern Baltic cod. However, the new method needed to be validated.

Mg:Ca and P:Ca are the chemical ratios considered physiologically regulated, reflecting metabolic status and growth. These received the highest scores, while the environmental proxies Sr:Ca and Ba:Ca, received lower scores and Mn:Ca, an index for both environment (seasonal hypoxia) and physiology (growth) scored in between. A significantly higher mean ranking score for otolith image readability for cod otoliths from the North Sea confirmed that the eastern Baltic cod otoliths are more difficult to interpret. No significant differences between decades (1980s – 2010s) were found, indicating that otolith readability for eastern Baltic cod had been constantly low over time.

The image opacity of the annotated marks was quantified to investigate which structures visual readers selected as annuli and what they corresponded to chemically. The results showed declining opacity values with age in general. For annuli 1 and 2, the chemical method located the annuli in brighter zones with significantly higher opacity values. There was high agreement among readers due to very clear seasonal minima, especially in Mg:Ca and Mn:Ca. The first chemical annulus was often visible, whereas annuli 2 and 3 generally corresponded to opaque zones.

Juvenile cod in shallow, coastal habitats may be exposed to warm water and hypoxia during summer, resulting in translucent zones caused by stress (Fablet *et al.*, 2011; Høie *et al.*, 2009). These zones often corresponded to peaks in Mn:Ca, indicating hypoxic episodes, but were interpreted as annuli by visual age readers. For annuli > 4, the opposite pattern was seen with thinner, significantly darker zones. The shift to a more distinct periodic pattern in otoliths with age indicate a more consistent annual cycle for adults, undertaking seasonal spawning and feeding migration (Cohen, 1990; Uzars and Plikshs, 2000).

4.2 Studies of somatic and otolith growth in relation to otolith chemistry

Several studies have linked Mg:Ca to somatic growth (Miller and Hurst, 2020; Stanley et al., 2015; Sturrock et al., 2015). P:Ca has only recently been reported on as a physiologically sensitive trace element that appears to have promise for ageing (Hüssy *et al.*, 2020b; Hüssy *et al.*, 2020c). However, the combination of the two in relation to stress factors has not been investigated. In **Paper III**, two hypotheses were tested: that (1) otolith Mg:Ca and P:Ca increase proportionally with somatic growth and increasing body condition and (2) that stress factors such as temperature, food availability, liver parasite infection, and hypoxia exposure may influence otolith Mg:Ca and P:Ca.

4.2.1 Aquarium experiment

The cod otoliths from the public aquarium at Skansen (**Paper III**) gave me a unique opportunity to study somatic and otolith growth in relation to otolith chemistry under stable conditions concerning oxygen, salinity and temperature along with abundant, nutritionally balanced food. The samples also provided insights that eastern Baltic cod, transferred from deteriorating, hypoxic conditions in the Baltic Sea would grow and thrive when transferred to a new environment with more favourable conditions. Somatic growth in the aquarium at Skansen could be estimated by measuring otolith growth from visually clear check marks in cross sections (Fig.6) and distinct responses in trace elemental incorporation.

The cod grew between 14-179 mm in the aquarium, with greater growth for the fish from the second batch that had spent longer time in captivity. Corresponding otolith chemistry showed marked increases immediately after the cod entered the aquarium, especially large in P:Ca and Ba:Ca, moderate in Mg:Ca and Sr:Ca and little to none in Mn:Ca in comparison to past histories of hypoxia exposure indicated by otolith chemistry. Since the aquarium was oxygenated, the small increase in Mn:Ca indicated growth rather than hypoxia.

Mean Mg:Ca was positively related to fish growth, whereas mean Mn:Ca showed a non-significant trend. Although P:Ca increased rapidly with high levels when the cod entered the aquarium, it thereafter declined, resulting in a non-significant trend for mean P:Ca. The onset of marked change in otolith chemistry varied among the five ratios, with Mg:Ca changing more-or-less concurrently with the production of the visible growth check, P:Ca commencing shortly thereafter, but with the largest lags being observed in Sr:Ca.

4.2.2 Field experiment

The samples received from the field experiment outside Karlshamn in the southern Baltic Sea provided another opportunity to study chemical response to feeding, this time in eastern Baltic cod kept in sea pens. The findings of **Paper III** showed that the most parsimonious and biologically relevant model explaining changes in otolith Mg:Ca during the feeding experiment included cumulative daily mean temperature, liver worm infection intensity (LWI) and difference in length (ΔL), pre- and post-experiment.

The best model for P:Ca included the difference in Fulton's condition factor (ΔK) pre- and post-experiment and LWI. There was a positive correlation between Mg:Ca and ΔL as well as between P:Ca and ΔK indicating that growth during the experiment affected otolith biomineralization and uptake of these ratios.

The correlation between Mg:Ca, P:Ca and LWI, indicated that increasing numbers of parasites in the liver affected the chemical proxies of fish metabolic status negatively. This is in line with other studies, where Horbowy *et al.* (2016) showed that Fulton's condition factor decreased significantly with increasing numbers of liver parasites and the study by Marnis et al. (2019) showing that infection with liver worm was negatively associated with regulation of cod genes related to metabolism, immune system, and growth.

4.3 Validation - Daily increments

The new chemical age estimation method (**Paper II**) and the findings from the aquarium and field experiments (**Paper III**) were validated with a new successful age validating method developed by Hüssy *et al.* (2020b). This method was based on concordance of seasonal patterns in chemical profiles and annual growth zones, and validated by analyses of daily increments and chemically marked otoliths from tagged and recaptured Baltic cod (Hüssy *et al.*, 2020a).

The validation method was tested on otoliths from eastern Baltic cod caught in SD 25 in 2017 and 2018, chemically aged 1-3 years old.

I measured the distance from the otolith core to the point where the daily growth increments disappeared completely and called that annulus one. I repeated the procedure between annulus one and annulus two and where possible between annulus two and annulus three, validating annuli according to (Pannella, 1971). In the same manner as Hüssy et al. (2020b), I used a loess smoothing algorithm to identify concentration minima along the valleys and peaks in chemical profiles. Thereafter, the distances between chemical minima were compared to the distances between identified annuli.

Both Mg and P showed strong positive correlations with annual growth zones (0.89 and 0.88) and the results showed 50% agreement for Mg and 44% for P in comparison to the annual growth zones determined by assessing daily increments. The loess smoothing algorithm sometimes failed to identify chemical minima close to the otolith edge, which was the main reason for the low percent agreement.

When assessing the results in detail, I could observe that Mg often captured the first annulus better than P. I recognized this phenomenon, in fact, when I chemically age estimated cod otoliths included in this thesis I almost always used Mg:Ca for identifying the first and second annuli because it had clearer seasonal pattern. By the third or fourth year I would usually switch to P:Ca. At older ages Mg:Ca often declined and became increasingly difficult to interpret. It was the opposite case for P:Ca, which developed distinct large-amplitude seasonal growth patterns after the age of three (Fig. 9). Therefore, I would recommend using a sequential combination of Mg:Ca and P:Ca for chemically estimating age of the eastern Baltic cod.

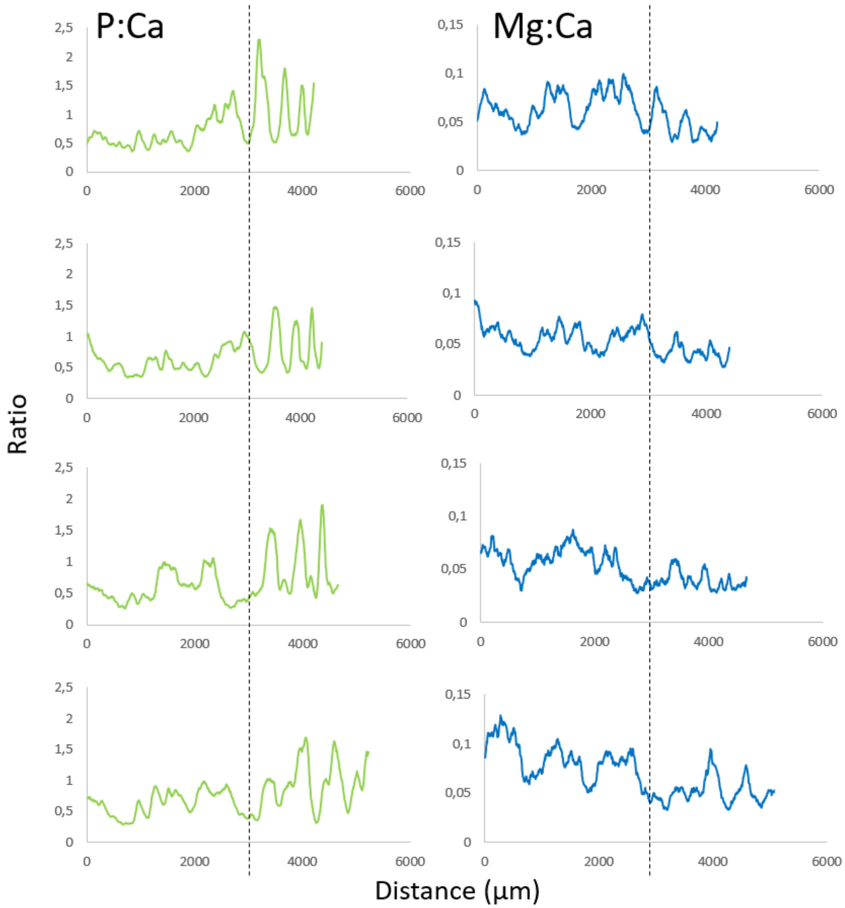

Figure 9. Chemical profiles illustrating variations in P:Ca (green) and Mg:Ca (blue), from the core (birth) to the dorsal edge (death). Seasonal patterns within the first three years of life are generally easiest to interpret from Mg:Ca, showing more distinct variations in concentration than P:Ca. For age > 3 (black vertical line), P:Ca generally begin to produce large-amplitude seasonal cycles, whereas Mg:Ca decline, with patterns more difficult to interpret. Reproduced from Paper III.

4.4 Tracking historic hypoxia exposure and metabolic status

Based on the long time series in **Paper IV,** I explored the concept of using the eastern Baltic cod as a sentinel species and their otoliths as bioindicators for tracking the accelerating climate change in the Baltic Sea over the last century (1927-2018). Focusing on hypoxia, the cod were compared to caged canaries in the coal mines, used as a warning system of low oxygen and toxic gases.

In order to eliminate the growth factor, Limburg and Casini (2018) developed a new proxy for hypoxia by dividing Mn by Mg (Mn:Mg). Mg is hypothesized to relate to metabolic activity and growth but not hypoxia (Limburg et al., 2018). Thus, by dividing Mn, which appears to incorporate as a function of environmental concentration of Mn^{2+} but also growth, by growth-sensitive Mg, Limburg and Casini tried to adjust for the growth effect. For age estimation purposes of EBC, Mn:Ca and Mn:Mg should be used with caution, partly reflecting growth within the first two annual growth zones to thereafter mainly tracking seasonal exposure to hypoxia which might vary considerably due to migration behaviour and major Baltic inflows (Heimbrand *et al.*, 2020; Hüssy *et al.*, 2016a; Hüssy *et al.*, 2020b).

By quantifying hypoxia, measured as mean within-year Mn:Mg it was possible to track historic annual hypoxia exposure. The proxy was elevated throughout modern samples, ranging from 1927 to 2018.

A normoxic baseline was created from otoliths found in an archaeological site on the island of Gotland in SD 27, representing the Neolithic baseline ~4500 years before present time (Fig. 10). From the 1930s to the 1960s, the annual hypoxia exposure varied within relatively stable levels, likely reflecting inflows of oxygenated water from the North Sea. Around 1975, however, the Mn:Mg levels increased dramatically, only briefly interrupted by the major Baltic inflow in 1993, but thereafter reaching the highest values of the last century in the 2010s. The increasing trend in Mn:Mg was directly correlated with anoxic and hypoxic volumes and indirectly to temperature, which exacerbates hypoxia as warmer water reduces oxygen solubility (Fennel and Testa, 2019). Increasing hypoxia exposure was confirmed for both juveniles and adults in habitats of different salinities, proxied by otolith SrCa.

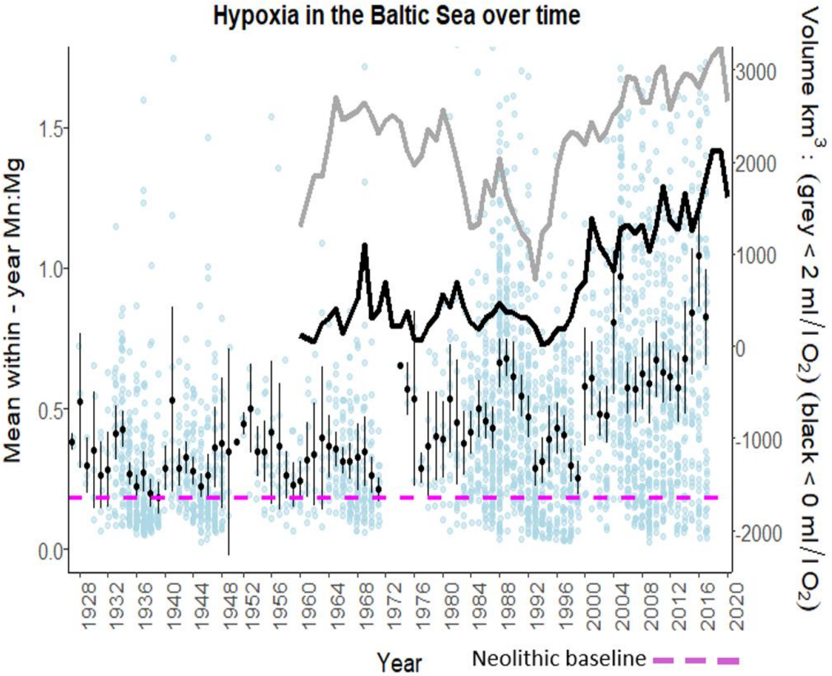

Figure 10. Mean otolith Mg:Mn within year ± se, compared to hypoxic (< 2 ml/l O_2) and anoxic (< 0 ml/l O_2) volumes (km^3) in the Gotland Deep. The normoxic baseline is based on Mn:Mg from Neolithic samples (dashed horizontal line) Reproduced from **Paper III**.

Age classes ≥ 1 year were used to specifically assess metabolic status, proxied by mean within-year Mg:Ca for cod in offshore habitats and avoid influence from juvenile habitats. The Neolithic baseline exceeded approximately 90% of the modern timeline. The 1930s to the 1970s indicated generally low levels, but in the 1980s, Mg:Ca increased with highest levels towards the end of the 1990s, followed by a decline during the 2000s and 2010s. Otolith Mg:Ca correlated positively with percent saturated dissolved oxygen at 60-80 m depth in the Gotland Deep. Increased levels during the 1980s and 1990s, reflect times of documented good growth and thereafter levels fluctuate and decline. With this in mind, I wanted to investigate if chemically back-calculated growth would reflect the trends in Mg:Ca over time.

The findings in **Paper II** showed that the traditional, visual age estimation method systematically overestimated ages, which would result in lower estimated length at age. By applying the new chemical age estimation method it was possible to predict chemically back-calculated historic mean length at age for the Eastern Baltic cod and to reconstruct past biological parameters from the unique time series as well as for Neolithic cod otoliths. The predictive model was based on repeated measurements on each otolith, by measuring the distance from the otolith core to each chemically assigned annuli in relation to total length at catch, taking into account random effects of individual fish. The model predicted highest growth in the 1990s and lowest in the 2010s, demonstrating a dramatic shift toward truncated, smaller size classes (see Fig. 11, below, and Fig. 6 in **Paper IV**).

Another recent study by Mion *et al.* (2021) using historical tagging data of eastern Baltic cod from the last seven decades also confirmed lowest growth in the 2010s compared with other decades. Otolith chemistry from the long time series reflects the worsening situation for cod in the Baltic Sea that can be likened to a coal mine for fish with expanding dead zones (Carstensen and Conley, 2019).

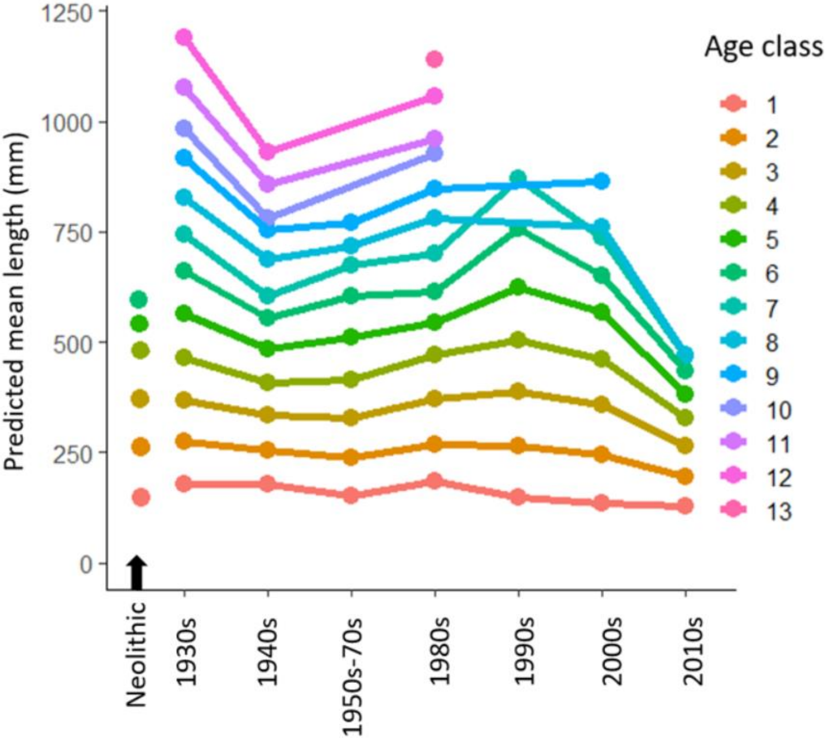

Figure 11. Predicted mean length per age of eastern Baltic cod over time, chemically back-calculated from otolith chemical annulus width from the core.

The findings in **Paper IV** highlighted ecological interactions and direct and indirect correlations between biotic and abiotic factors. I examined the correlations between extrinsic and intrinsic parameters affecting the eastern Baltic cod, with data covering 32 years, ranging between 1975-1977, 1980-1988, 1991-1997, 1999-2004 and 2011-2016, illustrated by Fig. 12, displaying only significant correlations.

The variables separate hierarchically into three groups depending on negative and positive correlations. Hypoxia formed one group with strong positive correlations between the abiotic drivers: temperature, anoxic and hypoxic volumes and otolith Mn:Mg. Metabolic status and growth were strongly linked with the abiotic factors: total nitrogen, total phosphorus and dissolved oxygen percent saturation and otolith Mg:Ca and P:Ca. The links between salinity, biomass of Baltic cod ≥ 35 cm (SD 24-32) and otolith Sr:Ca were positively correlated, but not studied specifically in this thesis. The correlation between the groups were mostly negative, except for otolith P:Ca and biomass for cod larger than 35 cm as well as for salinity, hypoxic volume and temperature > 100 m depth. The interactions between groups illustrate dynamic processes in the Baltic Sea, effects on cod and that otoliths can be used as bioindicators to provide information and fill knowledge gaps concerning the eastern Baltic cod.

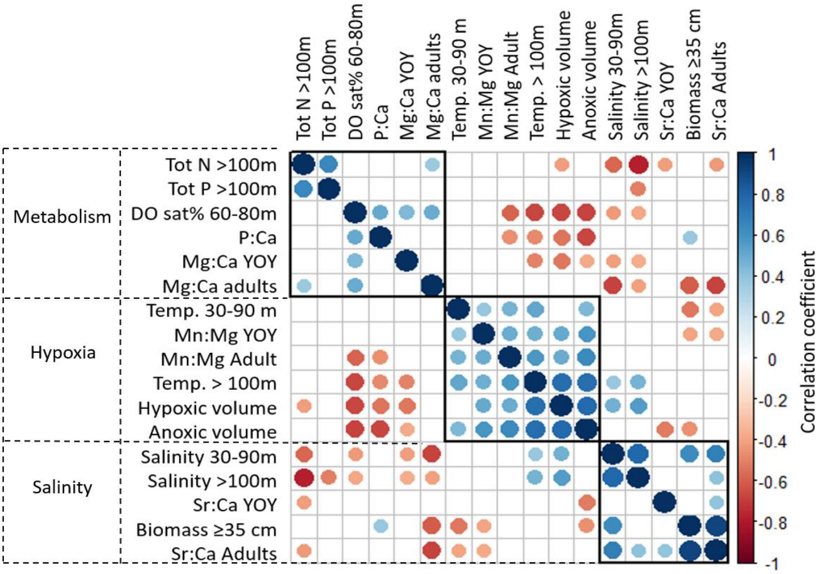

Figure 12. Correlation plot of significant extrinsic and intrinsic parameters affecting the eastern Baltic cod, including 32 years (1975-1977, 1980-1988, 1991-1997, 1999-2004 and 2011-2016). Otolith chemistry: Mean values of P:Ca per calendar year for all age classes. Mg:Ca, Mn:Mg and Sr:Ca for chemically back-calculated age class 0 (young of years, YOY) representing juvenile habitats and age classes > 0, considered to represent adult habitats. Abiotic variables: hypoxic (< 2 ml/l O2) and anoxic (< 0 ml/l O2) volumes (km3) in the Baltic Sea. Monitoring data from station BY15 in the Gotland deep, conducted by SMHI (the Swedish Meteorological and Hydrological Institute, https://sharkweb.smhi.se/) provided annual mean temperature and salinity at 30-90 m and >100 m depth, total nitrogen (Tot N), phorsphorus (Tot P) load µmol/l, and dissolved oxygen in percent saturation measured at at 60-80 m depth calculated according to (Garcia and Gordon, 1992) from annual mean oxygen (ml/l). Biomass (tonnes) of cod ≥ 35 cm in subdivisions 24–32 (ICES, 2019d).

5. Conclusions

This thesis addressed the problematic age determination of the eastern Baltic cod. The combined results present a solution based on the observation that the physiologically regulated trace elements magnesium (Mg) and phosphorus (P) display seasonal patterns reflecting metabolic status and growth that can be used for age determination.

Magnesium generally displayed the clearest seasonal pattern up to the age of three, thereafter concentrations declined and the pattern often became more difficult to interpret. Rarely measured by other researchers, phosphorus provided remarkably good seasonal patterns after age three, with distinct large-amplitude annual cycles.

The new chemical method proved to be a better alternative for ageing eastern Baltic cod than the traditional, visual method and was validated by studies of endogenous (growth, condition) and exogenous (temperature, parasites) factors. The chemical timeline of eastern Baltic cod from the 1930s to the 2010s and the Neolithic period provided insights in past environmental conditions experienced by individual cod. By assessing correlations between environmental parameters and otolith chemistry it was evident that otolith Mn:Mg was directly affected by hypoxia exposure and indirectly by water temperature. Increasing levels of Mn:Mg, especially in the 2010s reflected the intensifying hypoxia and anoxia in the Baltic Sea.

The proxy for metabolic status, Mg:Ca was positively correlated with oxygen saturation indicating that cod is a hypoxia-sensitive species. The 1980s and the 1990s were time periods with highest documented growth which was reflected by increased levels of Mg:Ca in otoliths representing these time periods. The reported decline in size structure from the 1990s to the 2010s, representing the lowest growth of all time could also be observed in chemically predicted mean length at age.

Overall, this thesis has provided a new otolith chemical method for age determination of the eastern Baltic cod, crucial for contributing validated age information to fisheries management for analytical assessments and scientific advice. The method also enabled reconstruction of historic environmental and physiological changes experienced by the fish on an annual scale which was applied on archived otoliths to better understand ecological processes in the Baltic Sea over time and the impact of climate change. This method holds promise to open up new avenues for research in age based processes such as timing of life history events and trophic interactions both in Baltic cod as well as other species.

6. Future perspectives

Several trace elements were tested when the new chemical age estimation method in **Paper II** was developed. Zinc was not suitable for aging Baltic cod in this study, but has been suggested to display seasonal growth patterns in otoliths from the Salmonidae, Esocidae and Gadidae families (Friedrich and Halden, 2010; Halden *et al.*, 2000; Limburg and Elfman, 2010). In order to expand the methodology it is important to develop species-specific chemical age estimation methods.

In this thesis, my results have described the eastern Baltic cod as a fish stock in distress. In the future, hypoxia and temperature are predicted to increase, while salinity is predicted to decrease (Meier *et al.*, 2012), putting even more stress on this marine species.

However, in the waters surrounding the Åland Islands, there is a glimmer of hope, where catches still include large sized cod in good condition. The cod thrive in the deep cold water with favorable oxygen conditions all the way down to the seabed at 200 – 300 m depth where the benthic fauna is healthy. Low salinity is the limiting factor for successful spawning in this area. However, new studies suggest that fertilization and egg development might be possible in lower salinities (9 – 10 PSU) than previously thought (Ulf Bergström, SLU, personal communication). Unfortunately, the salinity in the Åland Sea is below 7.5 PSU. Provenance and migration studies for determining the origin of these cod have become of interest. Can the eastern Baltic cod evolve and adapt to lower salinities in the future?

The trace elemental ratio Sr:Ca is often used in migration studies and Limburg et al. (2011) consistently observed low Sr:Ca in the inner otolith portions of cod from the Stone Age to the present time, and interpreted this as use of lower salinity nursery habitats. When I examined Sr:Ca in age class 0 in the otoliths from the 1930s to the 2010s, included in **Paper IV**, I found no significant differences in mean otolith Sr:Ca when comparing cod caught in the Bornholm Basin (SD25) and the Gotland Deep (SD28), indicating a preference for juvenile habitats with low salinity regardless of area (Fig. 13). The Neolithic otoliths reflect the higher salinity during that time period (Gustafsson and Westman, 2002).

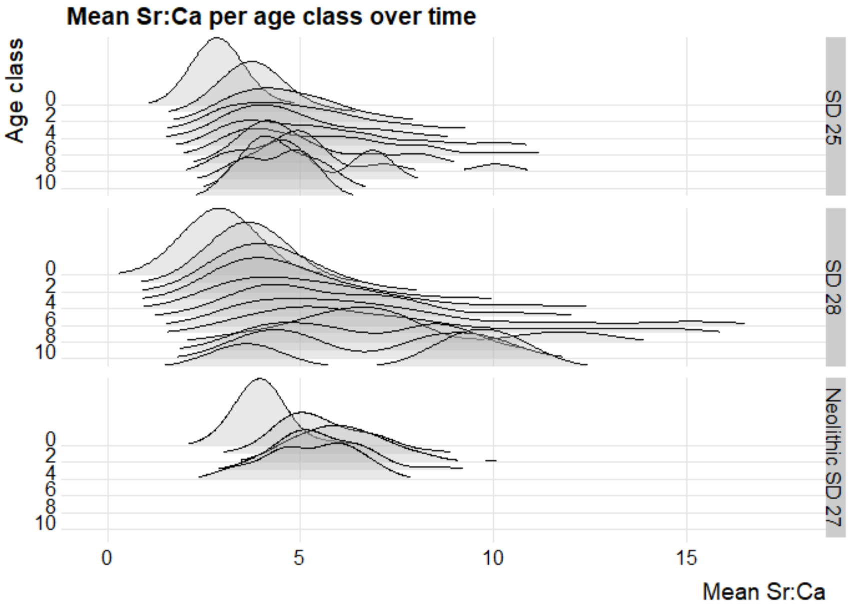

Figure 13. Distribution of mean Sr:Ca per area and chemically back-calculated age class. The mean Sr:Ca in SD 25 for age 0 (mean=2.87, S.D. = 0.49, C.I. 2.83, 2.92) and SD 28 (mean = 2.91, S.D. = 0.31, C.I. = 2.87, 2.95) was not significantly different, whereas SD 27 (Neolithic time period: mean= 3.99, S.D. = 0.55, C.I. = 3.83, 4.15), was significantly different to both SD 25 and SD 28 (F 2,789=83.6, R^2=0.173, $p > 0.05$).

It could therefore be difficult to determine provenance only by assessing mean Sr:Ca within the first year. Instead, detailed analyses on a finer scale combining different trace elements, isotopic ratios or genetic markers in combination with stable isotopes could be alternative methods for assessing origin and population structure (Brennan *et al.*, 2019; Brophy *et al.*, 2020; Rohtla *et al.*, 2017). The range of Sr:Ca increased with age and bimodal distribution for older age classes with a wider distribution in mean Sr:Ca indicated migration between habitats with different salinities (Fig. 13). The salinity gradient in the Baltic Sea range from north to south, but also vertically and varies depending on inflow conditions from the North Sea. It is therefore important to combine the use of Sr:Ca in migration studies with other elements and include environmental information of temporal and spatial changes in salinity, temperature and hypoxia over time.

A final recommendation for future fisheries research is to take advantage of all the otoliths that have been and continue to be collected for routine age determination purposes and saved in archives. With new technology, these remarkable small, calcified treasures with chronometric properties contain detailed information of the fish’s life history, a valuable aid in future management, chemical age validation and restoration of fish populations.

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Popular science summary

Climate change and human caused pressures affect virtually all ecosystems and their species worldwide. Excess nutrient runoff from the land and warming have led to a dramatic loss of oxygen in oceans, estuaries and coastal seas causing changes in the fish communities and within the populations that compose them. Time and seasonal cycles shape ecological interactions and processes. Age is therefore a fundamental parameter in biology and fishery science to estimate growth, age at maturity and mortality. Losing track of time, in the sense of lacking reliable estimates of age and growth, creates substantial problems for the management of species difficult to age.

Fish age is routinely determined by examining calcified structures called otoliths, situated in the inner ear. With structures similar to annual rings in tree trunks, otoliths produce annual growth zones in form of alternating opaque and translucent rings, corresponding to fast growth in summer and autumn and slow or no growth in winter. The number of growth zones corresponds to the age of the fish.

The cod is a key predator in the Baltic Sea and historically was an important commercially harvested fish species. The cod population in the eastern Baltic Sea (EBC) reached a peak in biomass in the 1980s, then experienced a drastic decline, driven by overfishing and deteriorating environmental conditions. The poor status of the cod resulted in smaller fish with reduced growth, body condition and size at maturity. Otoliths became increasingly more difficult to interpret due to the low degree of contrast between the annual growth zones and failed efforts to solve errors in age and growth estimations led to a situation where age-based stock assessment could not be performed between 2014 and 2018. Thereafter tagging studies provided a short-term solution to assessment, but in 2019 the eastern Baltic cod fishery was closed by the EU-Commission. Nevertheless, better methods for age determination were sorely needed.

The overarching theme of this thesis was to find a solution for the problematic determination of Baltic cod age. I developed a new method based on interpreting seasonal patterns in chemical profiles with quantified concentrations of trace elements from the otolith core (birth) to the edge (death). Trace elements reflect both environmental conditions in the surrounding water and fish physiology.

Previous studies had indicated that magnesium (Mg) was physiologically regulated and related to growth. Mg in otoliths from different fish species were examined and a hypothesis formed: that Mg reflected metabolism and growth. Therefore the seasonal patterns in Mg could be used to age fish, with high values in summer and low values in winter. By counting winters, the fish age is determined. To test the method, a set of cod otoliths were age determined by six highly experienced age readers using the traditional method of counting visual growth zones and by three readers interpreting otolith chemical profiles, based on ecological knowledge of environmental and physiological influences on otolith chemistry.

Mg and phosphorus (P) were the trace elements best displaying seasonal signals in otoliths. The new chemical method gave lower age error and higher agreement among readers than the traditional method. These results were promising, but validation was needed.

Instead, a three-step approach based on wild and captive fish was used to study how Mg and P responded to factors affecting growth. First, I analysed body and otolith growth in EBC transferred from the sea to an aquarium at the Baltic Sea Science Center at the Skansen Museum in Stockholm, Sweden. The cod thrived in the clean water and with abundant food they grew, but due to eye infection, a few of them had to be removed. The otoliths of these fish were chemically analysed.

Second, otoliths from a feeding experiment, that maintained EBC in sea pens along the coast in the southern Baltic Sea were also chemically analysed. These cod were individually tagged and it was therefore possible to measure changes in length, weight and condition pre- and post-experiment. The estimated body growth corresponded with otolith chemistry. The results showed higher Mg levels with increased length and higher P levels with increased body condition. The number of liver worms found in cod after the feeding trial, was negatively correlated with Mg and

P, indicating that the metabolic status and growth were affected by the amount of parasites.

Otoliths deposit not only annual, but also daily increments and in the third step it was possible to validate the annual widths with corresponding patterns in both Mg and P. Mg was better at identifying annual growth zones than P in young EBC. In older individuals however, P generally begins to display large amplitude seasonal cycles at age approximately > 3 years.

My recommendation, based on the studies in this thesis, is therefore to use a combination of the two, and use Mg for the first three years and thereafter P in chemical age determination of EBC otoliths.

To provide a historical perspective, I expanded my dataset and included a near century long data set of eastern Baltic cod otoliths as well as a rare collection of otoliths from the Stone Age. By chemically aging the otoliths, it was possible to separate the chemistry per calendar year and follow annual variations in manganese (Mn), reflecting hypoxia exposure. Mn was used to track hypoxia exposure over time and with the samples from the Stone Age, a baseline for normal oxygen conditions to assess changes over time. The results correlated with environmental data on changes in hypoxia and water temperature, indicating increasing effects of environmental stressors since the middle of the 1970s, with the highest levels in the 2010s. Mg was used to predict metabolic status and correlated with good growth during the 1980s and 1990s, declining thereafter. The relation between otolith annulus width and total length of the fish made it possible to back-calculate and predict historic mean length at age. The predicted mean length at age showed highest growth in the 1990s and a dramatic decline during the 2010s compared to all other time periods.

In summary, the results in this thesis contribute to increased biological knowledge of the EBC by providing a new method for determining age and growth parameters, to be used in age-based stock assessment and improve the scientific advice for fisheries management. The chemical method can also be used on other difficult-to-age fish species, although there may be species-specific differences. The utility of otoliths as chemical markers, providing data on an annual level is shown by the long time series of otoliths from the Stone Age and the last century. This chemical timeline provides unique information of how environmental processes and climate change affect individual fish and how that scales up to the population. Such information is essential for predicting future changes in fish population structure and thus for ecosystem-based management.

Populärvetenskaplig sammanfattning

Klimatförändringar och mänsklig inverkan på miljön påverkar akvatiska ekosystem och arter världen över. Syrebrist i haven, övergödning och överfiske påverkar fiskpopulationer negativt genom förlust av livsmiljöer för lek- och föda. Detta orsakar förändringar i fisksamhällets storleks- och ålderssammansättning. Tid och säsongsmässiga cykler påverkar ekologiska interaktioner och processer. En fisks ålder är därför en grundläggande faktor i fiskeriforskning och fungerar som en viktig indikator på tillväxt, ålder vid könsmognad och dödlighet. Felaktig uppskattning av ålder kan därför orsaka stora problem för fiskeriförvaltningen.

Åldern på fisken bestäms rutinmässigt genom att undersöka små hårda kalkstrukturer som kallas otoliter som finns i innerörats hörsel och balansorgan. Likt årsringarna i en trädstam, producerar otoliter årliga tillväxtzoner. Ogenomskinliga, vitaktiga, breda ringar motsvarar snabb tillväxt under sommar och höst och genomskinliga, tunna ringar motsvarar långsam tillväxt under vintern. Genom att räkna antalet vinterzoner kan man uppskatta fiskens ålder.

Torsken är en rovfisk med en viktig roll i Östersjöns ekosystem och historiskt har torsken varit en viktig art för yrkesfisket. Torskbeståndet i den östra delen av Östersjön var rekordhögt i början av 1980-talet, följt av en drastisk nedgång på grund av överfiske och försämrade miljöförhållanden. Torskens allt sämre hälsostatus har resulterat i att beståndet nu består av allt mindre fiskar med reducerad tillväxt, kroppskondition och storlek vid könsmognad. Det har blivit allt svårare att tolka det östra torskbeståndets otoliter på grund av låg kontrast mellan de årliga tillväxtzonerna. Misslyckade ansträngningar för att lösa problemen med ålders- och tillväxtuppskattningar ledde till att ingen åldersbaserad beståndsuppskattning kunde utföras mellan 2014 och 2018. Därefter fungerade märkningsstudier som en kortsiktig lösning för att utföra beståndsuppskattning. Läget blev allt mer kritiskt och 2019 beslutade EU-kommissionen om ett fiskestopp som en akut åtgärd för att rädda det östra torskbeståndet i Östersjön.

Det övergripande syftet med denna doktorsavhandling är att hitta en lösning för den problematiska åldersbestämningen av östersjötorsken. En ny metod utvecklades baserad på tolkning av säsongsmässiga mönster i upptaget av spårämnen i otoliten, från otolitkärnan (fiskens födelse) till kanten (fiskens död). Dessa spårämnen återspeglar både miljöförhållanden i det omgivande vattnet och förändringar i fiskens fysiologi. Tidigare studier har visat att magnesium (Mg) är relaterad till tillväxt och fysiologiskt reglerat. Därför undersöktes Mg i otoliter från olika fiskarter med en hypotes, att säsongsmässiga mönster i Mg-koncentrationen i otoliter kan användas för att bestämma och validera ålder av fisk.

För att testa metoden åldersbestämdes en uppsättning torskotoliter av sex erfarna åldersläsare som räknade antalet synliga tillväxtzoner enligt den traditionella metoden och tre åldersläsare som tolkade motsvarande otolitkemi, baserat på ekologisk kunskap om hur miljö och fysiologi påverkar upptaget av spårämnen i otoliter. Mg och fosfor (P) var de spårämnen som bäst visade säsongsmässiga signaler i otoliterna. Den nya kemiska metoden gav bättre noggrannhet och högre överensstämmelse bland åldersläsare än den traditionella, visuella metoden. Dessa resultat var lovande men behövde valideras.

För detta ändamål användes en strategi bestående av tre delar. Först bedömdes kroppslig tillväxt och otolittillväxt i östersjötorsk flyttad från havet till ett akvarium vid Östersjöns hus på Skansen i Stockholm. Torsken trivdes i det syrerika vattnet i akvariet och med riklig mattillgång började de att växa. Tyvärr drabbades vissa torskar av en ögonsjukdom och avlivades. Otoliter från dessa fiskar användes i syftet att analysera kopplingar mellan otolitkemi och kroppslig tillväxt.

I det andra steget utfördes kemianalyser på otoliter från ett fältexperiment där burfångad torsk från det östra beståndet i Östersjön matades med lokal sill för att se om torsken led av födobrist. Dessa torskar var individuellt märkta och det var därför möjligt att uppskatta förändringar i längd, vikt och kroppskondition före och efter experimentet. Otolitkemianalyser visade att koncentrationen av Mg ökade med tillväxt i längd och att P ökade med förbättrad kroppskondition. Det fanns även en negativ koppling mellan antalet levermaskar och koncentrationen av Mg och P, vilket tyder på att metabolismen påverkas negativt av mängden parasiter. Otoliter bildar inte bara årliga, utan även dagliga ringar. I det tredje och sista steget användes dagliga ringar som valideringsmetod för att bedöma hur väl det säsongsmässiga mönstret i Mg och P stämde överens med avståndet mellan årsringarna i otoliterna. Mg var det spårämne som var bäst lämpat för att identifiera de tre första åren. P var mer användningsbar för åldrar över tre år med tydliga säsongsmässiga cykler med större amplitud än Mg som över tid minskade i koncentration och ofta fick ett mer svårtolkat mönster med ökad ålder.

Rekommendationen, baserad på studierna i denna doktorsavhandling, är därför att använda en kombination av de två spårämnena genom att använda Mg för att identifiera de första tre åren och därefter P vid kemisk åldersbestämning av östersjötorsk. För att testa om den nya metoden kunde tillämpas för att undersöka förändringar i miljö och fysiologi som påverkat det östra torskbeståndet ur ett historiskt perspektiv, analyserades otoliter från nästan hundra år bakåt i tiden samt en sällsynt samling otoliter från stenåldern. Genom kemisk åldersbestämning var det möjligt att beräkna hur stor mängd spårämnen som tagits upp av otoliten per år.

Spårämnet mangan (Mn) visade om fisken hade blivit utsatt för syrebrist över tid och i vilken omfattning. Torskarna från stenåldern ansågs inte ha blivit utsatta för syrebrist och halten av Mn i dessa otoliter kunde därmed användas som en baslinje för att bedöma när torsken började exponeras. Det fanns ett samband mellan syrebrist, vattentemperatur och halten av Mn i otoliterna som indikerade att torsken hade lidit av syrebrist i allt högre grad sedan mitten av 1970-talet, och allra mest under 2010-talet. Spårämnet Mg användes för att avgöra metabolisk status och det fanns ett samband med den goda tillväxten under 1980- och 1990-talet som återspeglades av förhöjda värden i Mg som sedan avtog med allt lägre tillväxt. Relationen mellan otolitens längd och fiskens längd användes för att tillbakaräkna och uppskatta historisk genomsnittlig längd vid ålder. Resultaten visade högst tillväxthastighet på 1990-talet och därefter en dramatisk minskning under 2010-talet jämfört med alla andra tidsperioder.

Sammanfattningsvis bidrar resultaten i denna doktorsavhandling till en ökad grundläggande biologisk kunskap om det östra torskbeståndet i form av en ny kemisk åldersbestämningsmetod för validering av ålder- och tillväxtparametrar. Dessa parametrar bildar ett viktigt underlag för fiskeriförvaltningen i utförandet av åldersbaserade beståndsuppskattningar och för vetenskaplig rådgivning. Den kemiska metoden kan vara lämplig att använda på andra fiskarter som är svåra att åldersbestämma, även om det kan finnas artspecifika skillnader gällande t.ex. vilka spårämnen som är lämpligast att analysera.

Resultaten från den långa tidsserien av otoliter från stenåldern och det senaste århundradet visade att otoliter kan tillhandahålla information om fiskens livshistoria och miljön även bakåt i tiden. Återskapandet av historisk information ger en unik inblick i hur miljöprocesser och klimatförändringar över tid har påverkat enskilda fiskar. Sådan information är viktig för att förutsäga framtida förändringar på populationsnivå och för en ekosystembaserad förvaltning.

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Climate change and human activity profoundly affect aquatic ecosystems, pressuring fish populations via loss of essential habitat, disrupting all ecological levels. Time sets the context to understand and predict interactions and processes. Age is, therefore, necessary to assess vital rates and health of fish populations. "Losing track of time" in difficult-to-age species poses major challenges to sustainable fisheries management. A new aging method, using seasonal patterns in fish otolith chemistry, was successfully validated, and application demonstrated its promising utility.

Yvette Heimbrand received her Bachelor of Science degree within Biomedical laboratory sciences at Uppsala University in 1997. She has worked since 2006 within age and growth related fisheries research in the Baltic Sea.

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