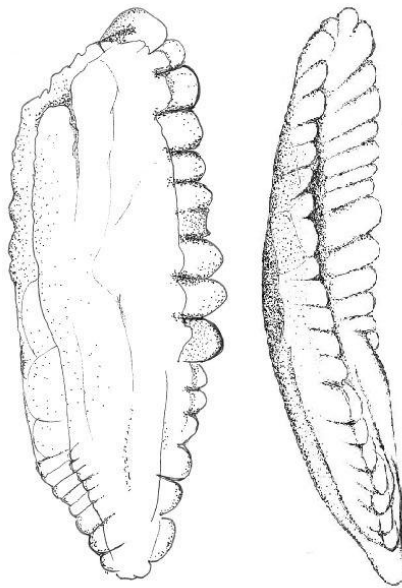# Unlocking the potential of biological sample archives:

benthic-pelagic feeding of Baltic cod assessed by otolith protein amino-acid specific stable isotope analysis

Bachelorarbeit

im Ein-Fach-Bachelorstudiengang Biologie

der Mathematisch-Naturwissenschaftlichen Fakultät

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LIST OF ABBREVIATIONS

δ	delta
AA	amino acid
Ala	alanine
Asx	asparagine
BB	Bornholm Basin
C	carbon
CSIA	Compound-Specific Stable Isotope Analysis
EAA	essential amino acid
Glx	glutamine
Gly	glycine
His	histidine
ICES	International Council for the Exploration of the Sea
Ile	isoleucine
ISOM	insoluble organic material
JFT	Jungfischtrawl
L	Carl von Linné
LDA	Linear Discriminant Analysis
LD1/LD2	linear discriminant 1/ linear discriminant 2
Leu	leucine
Lys	lysine
MD	mean difference
Met	methionine
N	nitrogen
n	samples size
p	p-Value
psu	particle salinity units
PCA	Principal Component Analysis
PC1/PC2	principal component 1/ principal component 2
Phe	phenylalanine
S	sulfur
SCA	Stomach Content Analysis
SD	Subdivision
sd	standard deviation
SIA	Stable Isotope Analysis
SOM	soluble organic material
Thr	threonine
TL	total length
Tyr	tyrosine
Val	valine

Zusammenfassung

Langzeitreihen sind wichtige Werkzeuge der Ökologen und werden eingesetzt, um Ökosystemveränderungen zu erklären. Da solche Datenreihen selten sind, wurden Methoden entwickelt, die rückblickend Analysen und die Rekonstruktion von Langzeitreihen ermöglichen. Otolithen sind Verknöcherungen im Innenohr von Fischen. Diese Strukturen werden seit Jahrhunderten in Museen und Forschungseinrichtungen gesammelt, da sie traditionell in Alterslesungen eingesetzt werden. Kürzlich wurde gezeigt, dass Otolithen potenziell eingesetzt werden können, um das Beutespektrum von Fischen zu rekonstruieren, was jedoch methodisch nicht einfach ist. In meiner Arbeit habe ich deshalb eine Kombination von zwei potentiell vielversprechenden Verfahren zur retrospektiven Analyse von Fischnahrung an den Otolithen von 12 Ostseedorschen (*Gadus morhua*), beprobt im Bornholmbecken, getestet: 1) stabile Isotopen Analyse am gesamten Gewebe (bulk SIA) mittels $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ und 2) Komponenten-spezifische stabile Isotopen Analyse (CSIA). Bei der CSIA werden aus dem zu untersuchenden Gewebe einzelne Komponenten, wie zum Beispiel die Aminosäuren (AA), extrahiert und einzeln analysiert, was die Auflösung von Ergebnissen im Vergleich zu bulk SIA Ergebnissen erhöhen kann. Bezüglich 1) bulk SIA erwies sich – auch wegen der besseren Verfügbarkeit von Rahmendaten von potenziellen Beuteorganismen – als die in der Ostsee momentan aussagekräftigere Methode für die retrospektiven Ernährungsanalysen von Fischen. Der Vergleich der Isotopenwerte der Otolithenprotein-Fraktionen mit den üblicherweise in Isotopenstudien verwendeten Muskelgewebeproben zeigte systematische und deshalb vorhersagbare Verschiebungen. Diese Verschiebungen spielen eine Schlüsselrolle für die Interpretation von Isotopenwerten der Otolithenproteine, wodurch die Anwendung der Verschiebungen auf die Isotopenwerte der Otolithenproteine theoretische Muskel-Isotopenwerte berechnet werden können, die anschließend mittels etablierter Interpretationsansätze für Muskel-Isotopenwerte gedeutet werden können. Hier zeigte die Interpretation der theoretischen bulk SIA Muskelwerte im Rahmen von Isotopenwerten möglicher Nahrungsquellen, dass die untersuchten Dorsche sich unabhängig von ihrer Größe pelagisch ernährten, d.h., ein benthisch-pelagischer ontogenetischer Nahrungs-Shift war nicht zu erkennen. Bezüglich 2), CSIA Resultate von essentiellen Aminosäuren im Otolithenprotein und in Muskelgewebe desselben Fisches waren

vergleichbar. Die Interpretation dieser Werte im Rahmen von – im Umfang begrenzten – Informationen zu möglichen Nahrungsquellen bestätigte teilweise die Bedeutung pelagischer Nahrung für Dorsch, wies aber auch auf eine potenziell in der Analyse fehlende Nahrungsquelle hin. Diese Studie unterstreicht das Potenzial der stabilen Isotopenanalyse von Otolithenproteinen für die Rekonstruktion der Nahrungsökologie von Fischen und bildet das Fundament für die zukünftige Rekonstruktion von Langzeitdatenreihen zur Bedeutung benthischer vs. pelagischer Nahrung für den Ostseedorsch.

Abstract

Ecologists often rely on long-term data series to explain and predict environmental change. Although such data sets are rare, records of past changes can be produced retroactively from materials such as otoliths, the inner ear structures or fish, archived in museums collections. These otoliths are formed over the life-time of a fish and are metabolically inert, and therefore sometimes considered a "black box" containing information about a fish's life. It can, however, be methodologically challenging reconstructing fish diets from otolith records. Here, in order to test the feasibility of the method, and to gain information about the feeding ecology of Baltic cod (*Gadus morhua*) from conserved otoliths, we applied a combination of two different stable isotope techniques to the protein component of otoliths of cod sampled in Bornholm Basin: 1) bulk stable isotope analysis (bulk SIA) on δ^{13}C, δ^{15}N, δ^{34}S isotopes and 2) compound specific stable isotope analysis (CSIA), which provides data on single compounds such as individual amino acids (AA) rather than total tissue and can enhance the resolution and improve differentiation between different food sources. Regarding 1), due to the currently better availability of diet framework data, we estimated the bulk stable isotope analysis to be the more powerful one for diet reconstruction purposes in the Baltic Sea. Bulk SIA comparison between otolith protein fractions and the traditionally used muscle tissue showed systematic and highly predictable shifts. These shifts played a key role for the interpretation of otolith protein isotope values. The application of our estimated shifts on the otolith protein isotope values resulted in theoretical muscle bulk isotope values, for which interpretation for feeding ecology purposes is established. Interpretation of these values in a framework of bulk SIA values of cod's potential prey indicated that Bornholm Basin cod showed an almost exclusively pelagic diet with no benthic - pelagic diet shift. Regarding 2), CSIA of essential amino acids yielded comparable results for otolith protein and muscle tissue of the same fish. CSIA results interpreted in a more limited framework of potential diet values in part confirmed the pelagic diet of cod, but also pointed to potential missing diet sources in the analysis. This study confirmed the potential of otoliths for retrospective analyses of the diet of fish and provides the foundation for the reconstruction of long-term data series in cod feeding ecology in the Baltic Sea using archived otolith collections.

Introduction

Ecosystems worldwide are affected by global change (Walther et al. 2002). The resulting changes in environmental conditions of sea and oceans include temperature increase (Levitus et al. 2000) and ocean acidification (Fabry et al. 2008). In addition, global change affects individual organisms, which results in fish migration (Walther et al. 2002) and whole organism communities changing their structure and functioning within ecosystems (Hoegh-Guldberg & Bruno 2010). The Baltic Sea (Fig. 1.) is one of the best known examples of an ecosystem where conditions for living organisms are getting more and more extreme influencing the organism's interactions (Österblom et al. 2007).

The Baltic Sea is one of the world's largest brackish water reservoirs and a semi-enclosed basin considered to be a low-diversity ecosystem (Thulin & Andrushaitis 2003). Global warming and environmental variability can potentially cause decreasing salinity and increasing sea surface temperature, which are expected to intensify by the end of this century (Österblom et al. 2007, ICES 2008). All that, and also the increasing eutrophication of the Baltic Sea, has an impact on the inhabitants of this environment, including fish populations (Pachur & Horbowy 2013, Carstensen et al. 2014). Environmental changes and likewise human activities, for example intensive fishing, resulted in the 1980s in a reorganization of the Baltic Sea from a cod (*Gadus morhua* L.)-dominated to a sprat (*Sprattus sprattus* L.)-dominated ecosystem (Österblom et al. 2007).
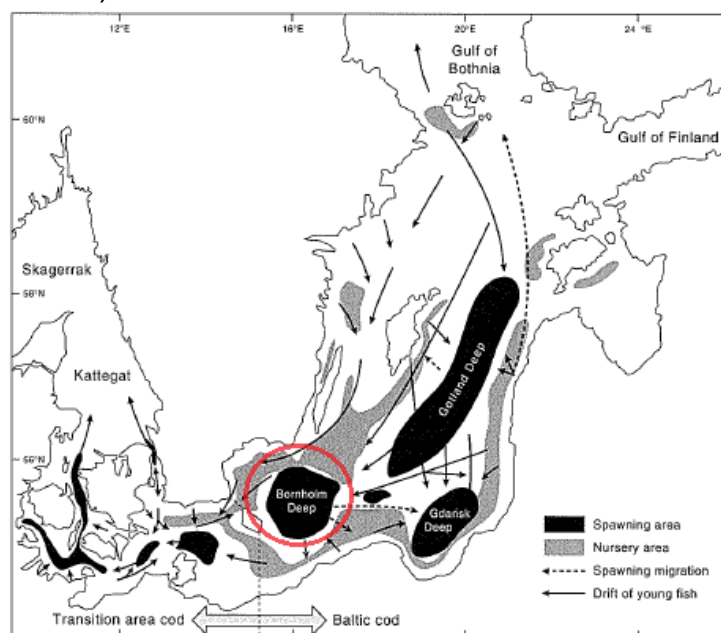
Figure 1. The Baltic Sea is a large brackish sea. The black spots indicate important sprawing areas for cod (*Gadus morhua* L.). The Baltic's important characteristics are low salinity, a salinity gradient from west to east (Kattegat: 33-37 psu, Bornholm Basin: 8-16 psu, Gotland Deep: 11 psu, Gulf of Bothnia: 1-3 psu) and eutrophication. The red circle shows the area studied in this thesis (modified from Bagge et al. 1994).

Baltic cod (*Gadus morhua*) is the most important top predator fish of the Baltic Sea, feeding both on benthic and pelagic prey (Bagge et al. 1994). It has been one of the species strongly affected by both climatic changes and high fishing
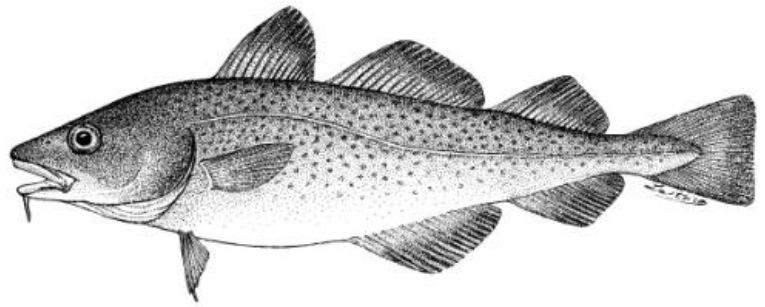
Figure 2. *Gadus morhua,* the Baltic's top predator fish. Because of the strong decrease in the eastern Baltic population of cod researches focus on detecting the causes of its decline (Cohen 1990).

pressure (Österblom et al. 2007). High abundance of the cod (Bagge et al. 1994) has begun to decrease after the Baltic regime shift in the 1980s, causing changes in the cod's feeding ecology. In addition, the regionally increased eutrophication led to larger oxygen-minimum and oxygen-hypoxia zones in the Baltic Sea, which in the end reduced the reproductive volume of cod eggs and its habitat (ICES 2008, Pachur & Horbowy 2013, Carstensen et al. 2014). It has been suggested that the decrease of benthic habitats observed in the Baltic Sea during the last decades may have led to a situation where the availability of benthic prey for cod is limited, resulting in changes in its feeding ecology (Rudstam et al. 1994, Möllmann et al. 2009, Casini et al. 2012). In particular, while in the past cod showed an ontogenetic shift from a benthic to a more pelagic diet, this shift may be weaker now (Eero et al. 2015, Mohm & Dierking *unpublished**), and in part explain the observation of declining cod condition since the 1990s (Eero et al. 2015). However, quantitative estimates of long-term dietary changes are missing.

Rapid changes within an ecosystem, including foodweb changes, are commonly poorly understood (Walther et al. 2002, Grønkjær et al. 2013). Long-term data sets of biological sample archives are the best for analysing such changes, but are rare (Grønkjær et al. 2013) due the fragility of organic material. Another opportunity for creating biological time series is the use of historical collections or conserved samples to retroactively create long-term data series. Hard materials like animal bones (DeNiro & Epstein 1978, 1981), scales (Wainright et al. 1993), teeth (Clementz & Koch 2001) and otoliths (McMahon et al. 2011, Grønkjær et al. 2013) and even plant materials, like trees (Bräuning & Mantwill 2004), resist degradation and therefore can be stored in museums or collections for long times. The only difficulties are in unlocking the information of these structures.
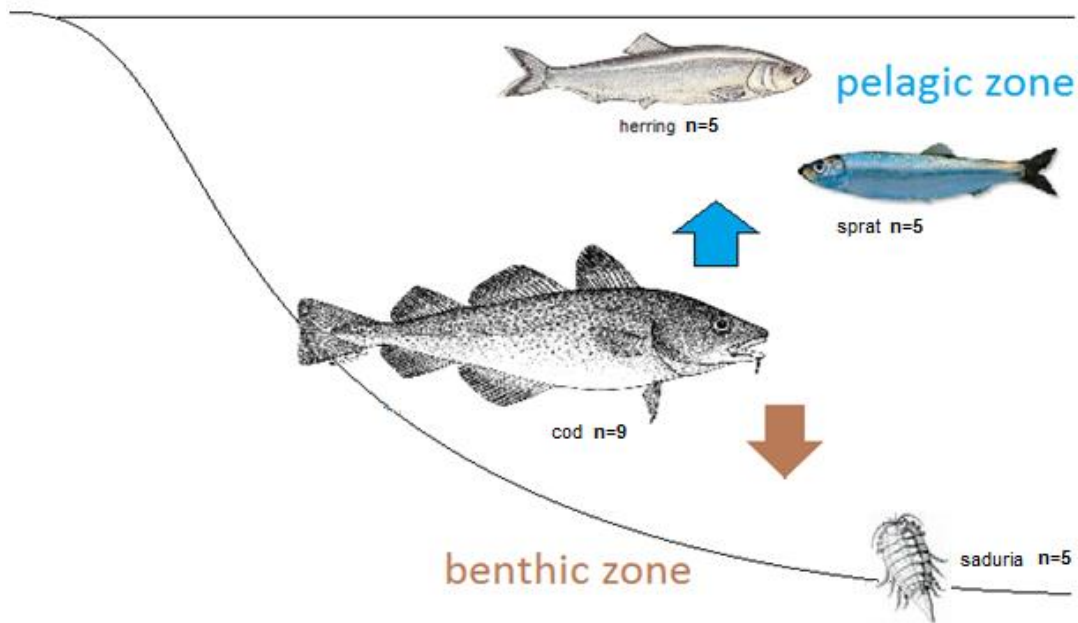
Figure 3. Schematic description of the cod's feeding ecology: the blue arrow shows the cod's pelagic food sources represented by herring and sprat, the brown arrow shows the cod's benthic food sources represented by saduria. The numbers behind the organisms represent the number of organisms used in this study, f.e." cod n=9" 9 cod individuals were used in this study.

Otoliths (sagittae) (Fig. 4.), calcareous inner ear structures of fish, have been termed "the black box" of fish because of the time-resolved information that they can provide about a fish's life and biology (Degens et al. 1969, McMahon et al. 2011, Grønkjær et al. 2013). Otoliths are gradually formed over the life-time of a fish, by accruing calcium carbonate around an organic protein matrix, otolin (Degens et al. 1969, Gauldie 1993, Grønkjær et al. 2013) as well as small amounts of different trace elements taken up from the environment (Kalish 1989, Marohn et al. 2011). The otolith is metabolically inactive i.e., once protein or organic material is settled down it cannot be further modified through metabolic processes (Rowell et al. 2010, Grønkjær et al. 2013). In the process, annual rings are added, which can be counted similarly to the age determination of trees (Campana 1999). Historically, since roughly the year 1900, otoliths have been therefore collected by fisheries resource management institutes for the ageing of fish, and collections are available for many fish stocks around the world.
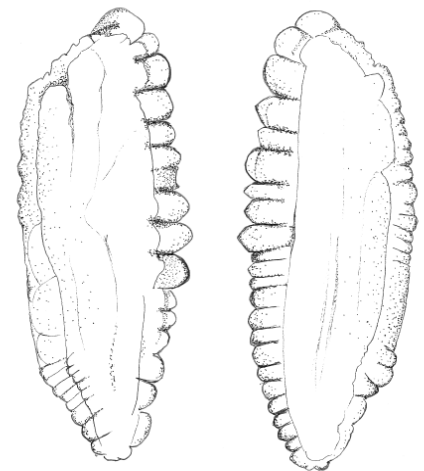
Figure 4. Pair of otoliths (sagittae) of *Gadus morhua.* Drawing of Jonas Mölle.

More recently otoliths attract more applications: otoliths shape, microchemistry profiles and stable isotope values of inorganic otoliths material (aragonite) can be used as a natural tag of nursery (Dierking et al. 2012), migration patterns (Campana 1999, McMahon et al. 2011) and stock structure (Campana & Casselman 1993). DNA from otolith surface can be used to reconstruct genetic time
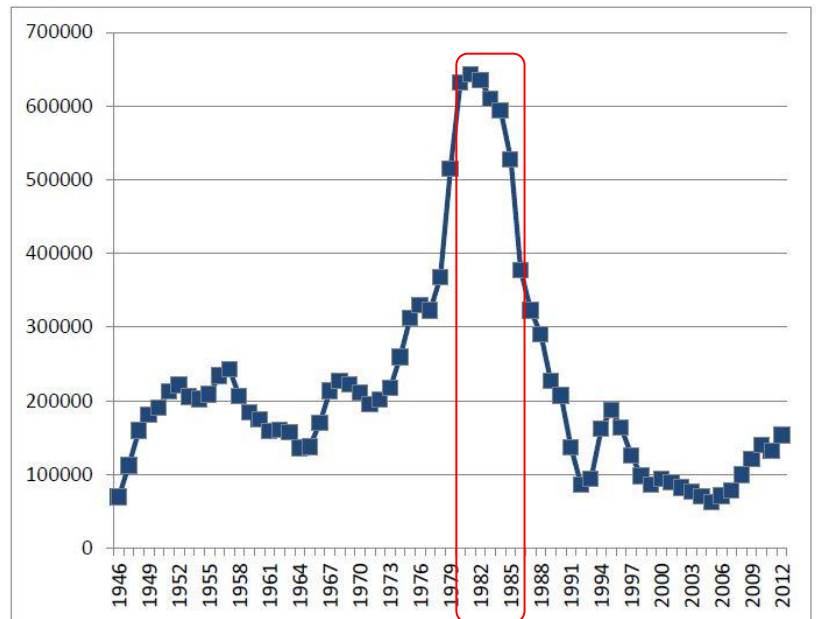
Figure 5. Graph of the decline of the eastern Baltic cod population after the regime shift in the 1980s (modified from (HELCOM 2013)).

series (Jakobsdottir et al. 2006) and address questions related to fisheries-induced evolution and population structure changes (Bonanomi et al. 2015). A novel application of otoliths has been developed even more recently, using the organic matrix of otoliths that presents roughly 0.2 to 10% of the otolith mass (Degens et al. 1969, Hüssy et al. 2004, Grønkjær et al. 2013). Pilot studies show the potential feasibility of using the organic component to reconstruct feeding ecology information, using stable isotope analysis (SIA) (McMahon et al. 2010, McMahon et al. 2011, Grønkjær et al. 2013). Those first approaches are big steps towards the application of otoliths to reconstruct time series of fish diet, but still more investigation and development in understanding of the results is needed.

Since the 1980s, stable isotope analysis (SIA) has become an essential approach to understand ecosystem functioning (Peterson & Fry 1987) complementing traditionally used stomach content analysis (SCA) (Hyslop 1980). SIA measurements are based on the stable nature of heavy and light stable isotopes of elements, and due to the predictable fractionation from one trophic level to the next. With each next trophic level heavier isotopes accumulate in the animal's tissue, therefore analysis of the isotopic depletion or enrichment of a tissue can reveal the trophic level (primary producer, consumer, top consumer) of the analysed individual, based on: "you are what you eat" (DeNiro & Epstein 1978, Fry 2006). $\delta^{15}N$ provides information about the trophic position.

On average, the pattern observed is: the higher is the trophic level, the more enriched the $\delta^{15}N$ values, with top predators. $\delta^{15}N$ is an almost pure diet record, because of the small fractionation from one trophic level to the next. $\delta^{13}C$ has been used for tracing pelagic (primary) producers and benthic organisms. Measurements of $\delta^{34}S$ are particularly powerful to distinguish between benthic and pelagic sources. $\delta^{34}S$ values are powerful tools for ecosystems like the Baltic Sea (Fig. 6.), where $\delta^{13}C$ is particularly limited because of its resolution. It shows some discrepancies in the distinction between different marine food sources (Peterson & Fry 1987, Fry 2006, Mittermayr et al. 2014, Mohm & Dierking *unpublished*).
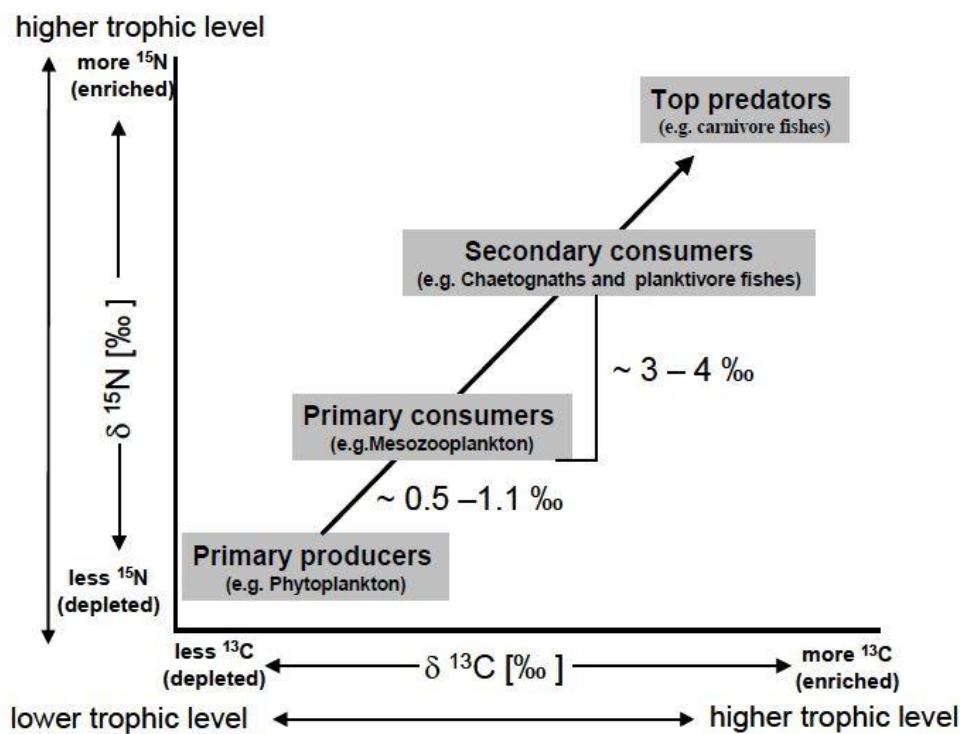
Figure 6. Schematic plot of $\delta^{13}C$ and $\delta^{15}N$ isotope enrichment/depletion indicating the trophic level within a food chain in a marine ecosystem (Agurto 2007).

There are two kinds of SIA, the bulk SIA i.e. the measurements of isotope ratio of tissues and the compound-specific stable isotope analysis, which measures the isotope value of certain components of tissues (Larsen et al. 2013, Larsen et al. 2015). The bulk SIA is now one of the most influential tools in ecological studies, but the quantification of the relative importance of different food sources in the long-term diet of a species can be difficult in particular if many food sources are present or if tissue-specific fractionation factors are not well understood (DeNiro & Epstein 1981, McMahon et al. 2011).

CSIA can overcome these obstacles. In the CSIA values of single components like, for example AAs, are measured, this leads to higher resolution that allows better distinction between multiple food sources (McMahon et al. 2011, Larsen et al. 2013). AAs, especially the essential AAs (EAAs), are much more independent of isotope baselines, whereas baselines of bulk SIA can vary strongly across different seasons and locations (McMahon et al. 2011, Larsen et al. 2013, Mohm & Dierking thesis 2014). Small fractionation factors (0 ‰ to 1 ‰) in essential AAs, and larger fractionation in non-essential AAs have been shown in previous bone collagen and otolith studies (O'Brien et al. 2002, Jim et al. 2006, McMahon et al. 2010), underlining the importance of separation AAs and at the same time advantage of the EAAs. Both bulk SIA and CSIA traditionally focused on soft tissues, e.g., muscles, of organisms and addressed one or few time points, but not long-term data series. Therefore analysis of organic material preserved out of hard structures such as bones or fish otoliths are a promising avenue to reconstruct the feeding ecology of organisms that lived and died a long time ago (DeNiro & Epstein 1978, 1981, Rowell et al. 2010, McMahon et al. 2011, Grønkjær et al. 2013, Wiley et al. 2013). A limiting factor for these analyses is the protein amount obtained from otoliths.

There is only a small proportion of protein in otoliths. The current method of the compound-specific analysis is not adjusted to such a small amount of material, so only cod of a defined size with big enough otoliths (otoliths mass > 150mg) can be analysed. Principle methods were addressed for wild fish and laboratory grown fish (Rowell et al. 2010, McMahon et al. 2011, Grønkjær et al. 2013) in order to estimate tissue-specific fractionation factors for otolith proteins bulk SIA using C and N, but never confirmed for S. A pilot data set on compound-specific stable isotope analysis (CSIA) for C was performed, comparing muscle CSIA and otolith CSIA, but not for the same individuals.

This study makes use of an existing cod otolith collection at GEOMAR, Helmholtz-Zentrum für Ozeanforschung Kiel in the Department of Evolutionary Ecology of Marine Fishes conducted as a part of the Baltic Sea integrative long-term data series since 1996. The goals are (1) to confirm the feasibility of otolith protein bulk SIA (C, N, S) and assess CSIA on this tissue, for the first time for a commercially important fish, and also to compare these results with analyses of the traditionally used muscle tissue.

(2) To use cod otolith protein bulk SIA (C, N, S) and CSIA data and place it into a framework of isotopic signatures of potential diet sources to assess the feasibility of the reconstruction of a long-term feeding ecology data series on Baltic cod for a better understanding of ecological shifts over the past decades (Fig. 5.). Our goal is to create a tool set for using otoliths like "time machines".

Hypothesis

Methodology

- How do bulk SIA values (C, N, S) differ between muscle tissue and otolith protein of the same cod individual?
- How do CSIA (C) values differ between muscle tissue and otolith protein?
- How do bulk C values and CSIA C values for the same individual in the same tissue differ?
- How strongly do bulk SIA values differ between left and right otolith proteins of the same fish?
- How strongly do bulk and CSIA values differ between different individuals of cod?

Biology

- How do bulk and CSIA muscle and otolith protein values of cod individuals compare to the values exhibited by potential diet organisms?
- Do the results support previous published results of cod feeding ecology based on muscle bulk SIA?
- Are there new / additional insights, e.g., regarding the ontogenetic shift in cod or individual feeding specialization?
- Is the application of cod otolith bulk SIA and CSIA on historic otolith collections feasible, and if yes, what would be the best way to proceed?

Figure 7. Hypotheses of this study presented as questions. This thesis consists of two sub-parts: methodology and biology. For each part individual questions were designed**.**

Material and Methods

Study area

The Baltic Sea is a semi-enclosed brackish water area (Fig. 1. & 8.). It is characterized by a vertical stratification on its water layers, and a long residence (turnover) time for full exchange of its water mass estimated at 25-30 years (Thulin & Andrushaitis 2003). These features are major factors for the natural environmental conditions of the Baltic Sea: low salinity (between 1-20 psu with an average of 7 psu) eutrophication, oxygen-minimum and oxygen-hypoxia zones. In terms of global changes these conditions are getting more and more extreme, challenging the Baltic organisms even more (ICES 2008, Pachur & Horbowy 2013, BalticSTERN 2014,

Carstensen et al. 2014). Inflows of oxygen rich, saline and therefore heavier water from the North Sea and the large impact of river fresh water results in stratification of the water column in the whole Baltic.

The timing of the inflow events is un-predictable and infrequent, due to the climatic variability. These inflows are estimated to be very important for the productivity and a general well-being of the biota (Carstensen et al. 2014).

In this study we will focus on one of the deep basins of the Baltic - the Bornholm Basin, SD 25 (Thulin & Andrushaitis 2003)(Fig. 8.). Bornholm Basin is very important for eastern Baltic cod recruitment (Fig. 1.) (Bagge et al. 1994, Tomkiewicz et al. 1998), and is the most important fishing ground in the Baltic Sea (Köster et al. 2003).
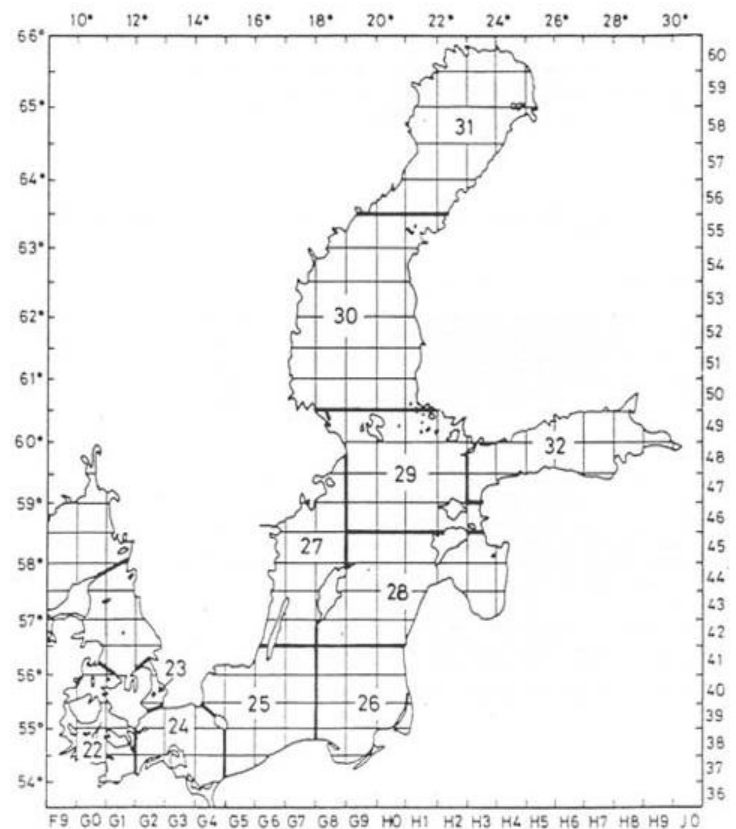
Figure 8. Baltic Sea divided in subdivisions (SD), which is necessary for management purposes. Fishes from SD 25 were examine in this study (ICES 2008).

Study organism

The Baltic Cod (Fig. 2.) is a representative of the Gadidae family, presenting a brown to greenish color with a light lateral line and a conspicuous barbell on its lower jaw (Cohen et al. 1990). It can reach up to 115 cm in the Baltic Sea, but such big examples are rare due to the high fishing pressure (Svedäng & Hornborg 2014). Cod plays a key role in Baltic ecosystem controlling the abundance of lower trophic levels via top-down control. Juvenile cod (<25 cm) have been described to prey on inverte-brates like *Mysis species*, *Pontopoeira species* and *Bylgides sarsi*, larger individuals (25-35 cm) on small clupeids (herring, sprat) and also benthic prey, especially *Saduria entomon* and larger fish (>35 cm) on pelagic organisms, clupeids like sprat (*Sprattus sprattus*) and herring (*Clupea harengus* L.) (Fig. 3.). Cannibalism also oc-curs and increases with the growth of the fish. Stomach content analysis has shown that benthic food sources are essential for cod diet (Bagge et al. 1994, Pachur &

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Horbowy 2013, Mohm & Dierking *unpublished*). The role of cod in the food web has changed after species regime shift. It has been suggested that the benthic prey has become less available for cod, resulting in enhanced preying on pelagic clupeids, but significant evidence could not be done so far (Eero et al. 2015).

Sampling

The sampling of fish was carried out on the research cruise AL 435 on the vessel Alkor in April 2014. All fishes used in this study were sampled in Bornholm Basin, ICES SD 25 (Fig. 8.), captured by pelagic trawls ("Jungfischtrawl", JFT) with a mesh size of 0.5 cm. We chose 12 cod individuals from 335 collected cod. Each cod was weighted (+/- 1g) and total length of the fish was measured, rounded to the next lower cm. Moreover, sex (male, female, juvenile) and maturity stage of each individual was characterized.

We then dissected and dried the otolith pairs (sagittae) (Fig. 4.) and measured their length, width, and weight. We differed between left and right otoliths and labeling each one differently.

From all these fishes muscle samples were taken carefully, without skin or blood, using a biopsy punch (4 mm; Stiefel; Durham,USA) or a scalpel. Samples were transferred into 2 ml polyethylene tubes (Sarstedt; Nümrecht, Germany), labeled and frozen immediately at – 20°C to avoid degradation process.

Sampling design

To best cover potential ontogenetic shifts in feeding ecology, we divided the collected cod by size in 3 groups: small (35-39 cm), middle (40-45 cm) and big (50-55 cm) fishes, and chose 3 representatives from each group for the study.

To assess the applicability of different stable isotope analyses on otolith protein we used two different approaches: bulk stable isotope analysis (bulk SIA) of carbon (C), nitrogen (N) and sulfur (S), and compound specific stable isotope analysis (CSIA) of C, on two different sample types: otolith protein and muscle tissue, which is the tissue used the most in studies applying stable isotope analysis for fish. To answer our hypothesis, we used the following approach:

I: Left otoliths of the 9 specimen were used for the compound-specific isotope analysis ($\delta^{13}C_{AA}$).

II: Right otoliths, also 9 however, for bulk stable isotope ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$).

III: 9 specimen for the muscle- tissue compound specific isotope analysis ($\delta^{13}C_{AA}$) and 12 specimen for the muscle bulk stable isotope ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$).

IV: Bulk stable isotope analysis ($\delta^{13}C$) of 3 different cod samples were used to compare left and right otoliths (Fig. 9.).
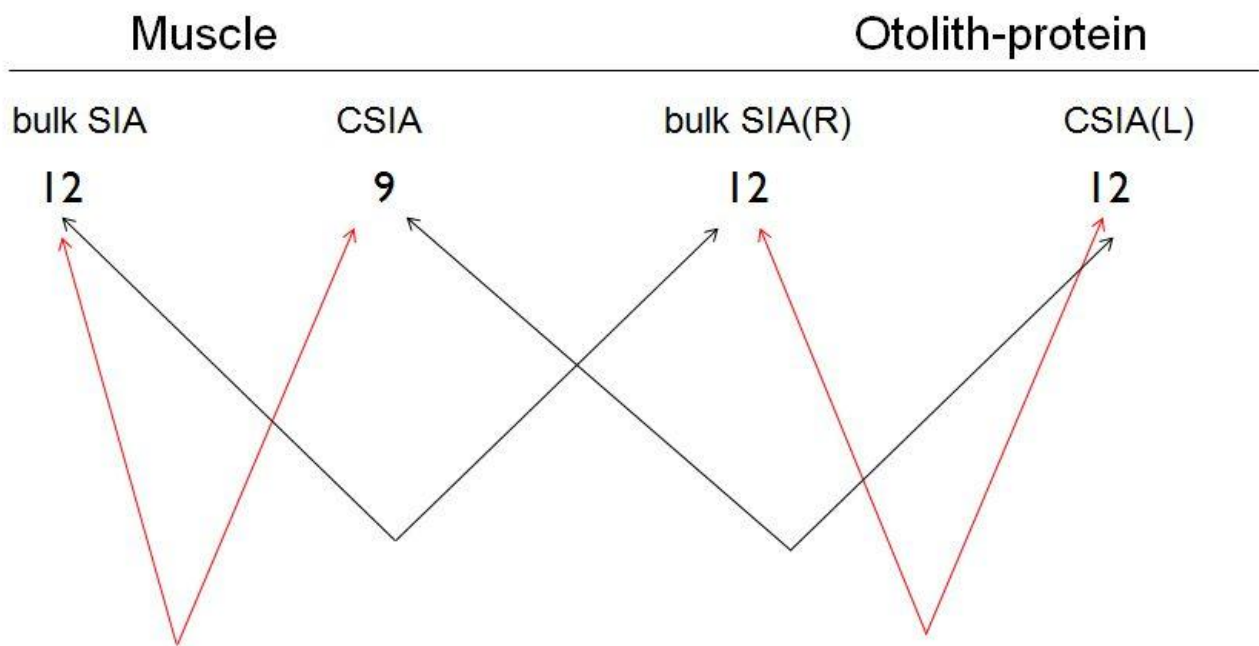
Figure 9. Sampling design for the methodology part. In this study our goal is to test the possible use of otoltihs for retrocative - reconstructions of feeding ecology of fishes. For that purpose we performed 2 approches for defining food sources by using otoliths of fish, comparing those to the common used muscle-tissue with known patterns. Number "9" symbolised the number of analysed individuals. Dark line represents 2 comparision ("Bulk"= bulk SIA) within the methods itself. Comparing same individuals but different tissues, the red line represends the comparision of the the 2 approches.

For both analyses, the bulk SIA and CSIA, we used the same specimen for a pair wise comparison. That will allow us a better evaluation of the feasibility of these 2 approaches and the best application for future studies.

Finally, to derive conclusions about cod feeding ecology from the bulk SIA and CSIA otolith protein results, with both methods (bulk SIA and CSIA) we created a framework by defining isotope values of benthic and pelagic prey (Fig. 3). The pelagic prey representatives used in the study were the clupeids: herring (*Clupea harengus* L.) (n=5) and sprat (*Sprattus sprattus* L.) (n=5), we studied also the benthic invertebrate prey *Saduria entomon* (n=5). From each prey we have chosen 5 repre-

sentatives for our analysis (Fig. 3.). In addition, a large framework of fish muscle tissue bulk SIA of C and N values was available from the same cruise from a parallel study on Baltic fish community feeding interactions (Mohm & Dierking *unpublished*).

Sample Preparation

<u>Extraction the organic matrix out of otoliths</u>

The analysis focuses on this organic matrix, the otolin. The extraction of the otolith protein is carried out in three steps, following Grønkjær at al.2013.

    <u>Cleaning</u> of otoliths is necessary to make sure that the obtained protein is only from the otoltihs and not from the adhered tissue. We transferred the otoliths into glass tubes and covered with O.2M NaOH for at least 30 min. After sonification for 1-2 min, we removed the rest of the adherent tissue by using a toothbrush. Finally, were washed those 3 times in Milli-Q water and dried out at 30°C for 48h. To improve the chemical process in the next step, the otoliths had to be grinded to a fine powder and transferred into 5ml microtubes.

    <u>Demineralization</u> was used to remove the inorganic carbon from the otolith powder. We transferred 2 ml of 5°C 0.1 mol·L^{-1} HCL into the microtubes with otolith powder inside. We stored the otoliths first at 5°C to start the demineralization. Once the demineralization started we placed the microtubes at room temperature for 1-2h. Afterwards we put the samples for 4-5 days into a heating cabinet to improve the demineralization process. Samples were then transferred into polyethylene test tubes.

    <u>Separation of the bulk organic material,</u> by centrifugation at 15 000 r·min^{-1} for 15min.The separation was improved by cleaning the test tubes with MilliQ water and centrifugation 3 times. The supernatant (SOM: soluble organic material) and the pre-cipitate (ISOM: insoluble organic material) separate visually. SOM was immediately transferred into cleaned Amicon Ultra filters. The HCL of the demineralization process was cleaned out from the SOM samples by adding 3.5 ml MilliQ water twice to the ultra filters centrifuged each time at 4000 r·min^{-1} for 2-3 min until the volume of the sample reached 50-100 µL. This volume we transferred into pre weighed poly-ethylene tubes.

The ISOM precipitate left in the microtubes was cleaned twice by adding 1ml MilliQ water each time into the tubes and centrifugation at 15 000 r·min^{-1} for 10 min. ISOM pellet was transferred into pre weighed tin vial using 70μL MilliQ water. The separation of the bulk organic material was necessary only for the bulk stable isotope analysis. Both samples were freeze-dried.

All this was performed by Peter Grønkjær Department of Bioscience, Aarhus University, Ole Worms Alle 1, 8000 Aarhus C, Denmark.

Muscle samples

All muscle samples were freeze-dried (freeze-dryer alpha 1-1; Christ GmbH; Osterode am Harz, Germany) and pulverized using a mortar and pestle. We then weighed 40-60 μg (MC 5 Micro Balance; Sartorius; Göttingen, Germany) of the powder into tin caps (3.2 x 4 mm) and added 25 mg ± 1 mg of Vanadium (V)-oxide.
We weighed 2.00 mg ± 0.10 mg of the muscle powder for CSIA into tin caps of 5 x 8 mm (Filter Scale SE2F; Sartorius; Göttingen, Germany). The muscle CSIA was performed in exactly the same way, like the otolith CISA. For bulk SIA we measured 40-60 μg.

We used the same specimen for otolith proteins and muscles as well as for both of the analyses in order to provide comparable results (Fig. 9. & 10.). All the steps of sample preparation for both analyses are visualized in Fig. 10.

Stable isotope analysis (SIA)

Principle of bulk stable isotope analysis (bulk SIA)

All elements occur in multiple forms, termed as isotopes - it means elements that differ in the number of neutrons, but have the same place in the periodic table (Fry 2006). In reactions different isotopes of one element behave slightly different, caused by isotope effects. An additional neutron does make the element more "heavier", resulting in different discrimination in comparison to the lighter isotope (Farquhar et al. 1989, Mittermayr et al. 2014).

The method can be applied for migration studies in aquatic (Michener et al. 2007, Dierking et al. 2012) and terrestrial (DeNiro & Epstein 1978, 1981, van der Merwe & Medina 1991) ecosystems, analyses of movement and stock structure for fisheries (Campana & Thorrold 2001) and diet studies (Mittermayr et al. 2014, Mohm & Dierking thesis 2014).

The fractionation factors (α) quantify isotope fractionation, it can be expressed by:

$$\alpha_{o\text{-}d} = R_o / R_{d,}$$

For muscle tissue α has been well investigated for many species: on average, the fractionation factor from one trophic level to the next is 0.5-1.5‰ for $\delta^{13}C$ (DeNiro & Epstein 1978, Peterson & Fry 1987, Sweeting et al. 2007b) and roughly ($\alpha=0.2$‰) for $\delta^{34}S$ (Peterson & Fry 1987) (Fig. 6.). For fish the specific $\delta^{15}N$ muscle enrichment factor has been assessed by approx. 3.2‰ relative to prey (Sweeting et al. 2007a). For cod juveniles enrichment factors for muscle (1.23‰ for $\delta^{13}C$ and 4.36‰ for $\delta^{15}N$) (Ankjærø et al. 2012) and otolith proteins (SOM-fraction: 0.14‰ for $\delta^{13}C$ and 0.51‰ for $\delta^{15}N$)(Grønkjær et al. 2013) where established.

Within an organism the isotope values can differ among different tissues. This phenomenon is called "routing" (Gannes et al. 1997). It can be caused by different turnover times of tissues or due different needs and the use tissue-specific nutrients by different tissue types. The consequence is that those tissues do not reflect exactly the same isotopic composition of the diet. In sum, in our measurements the resulting isotopic signature contains the mean isotopic value and the tissue specific fractionation factor (Gannes et al. 1997).
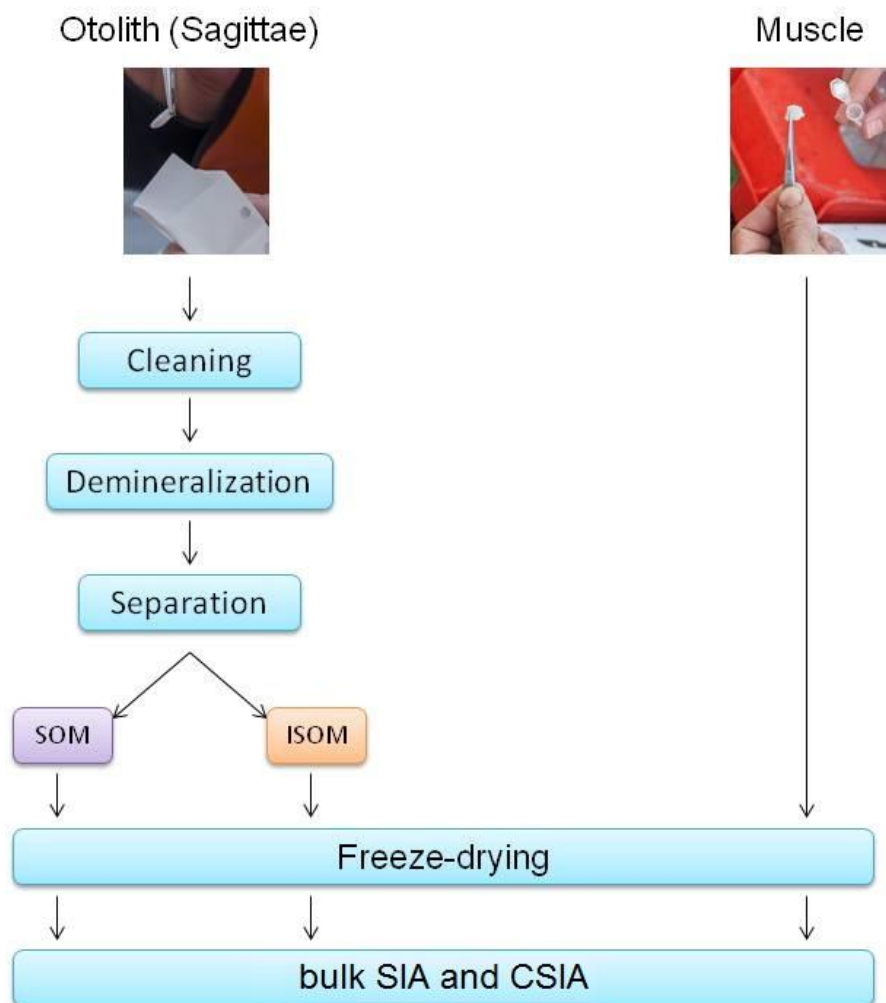
Figure 10. Diagram of the different steps for sample preparation. Soluble organic material (SOM), insoluble organic material (ISOM) and muscles are freeze-dried and analyses for either bulk SIA or CSIA separatly.

This study is meant to contribute more knowledge towards the interpretation of the isotope values of otolith-proteins. Therefore, we will assess the very well established way of interpretation of muscle isotope values on our findings and compare those to our otolith-protein results of the same individuals in order to find a way for understanding of the otolith-protein values.

For a correct estimation of the trophic position of the analysed organisms, it is necessary to analyse their potential prey. For this reason, we inserted cod's isotopic signature into a frame work of its diet. Then we then were able to reconstruct food sources of cod.

Stable isotope analysis of samples

With bulk SIA we measured the extracted organic matrix of the right otolith and fish muscle tissue. The otolith protein consist two fractions: soluble (SOM) and insoluble material (ISOM), which we measured separately from each other, because of reported significant differences in isotope values of these different fractions (Grønkjær et al. 2013).

For the measurements we weighed 30-50 µg of ISOM, 100-300 µg of SOM and 40-60µg of the muscle tissue. Each sample was weighed into 3.2 x 4 mm tin caps adding 25 mg ± 1 mg of Vanadium (V)-oxide short before folding the tin caps. Bulk SIA was carried out by continuous flow isotope ratio mass spectrometry (EA-IRMS/ GCC-IRMS) in the laboratory of Dr.Thomas Hansen at GEOMAR, Helmholtz-Zentrum für Ozeanforschung Kiel Experimentelle Ökologie I (Nahrungsnetze), Düsternbrooker Weg 20, 24105 Kiel

Samples were analysed and corrected for standards for ^{13}C, ^{15}N, ^{34}S .Stable isotopes are expressed in delta values ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$).

$$\delta X\ (‰) = [(R_{smpl}/R_{stnd})-1] * 1000‰,$$

where X is ^{13}C or ^{15}N or ^{34}S, R stand for the ration of heavy to light isotope an R_{smpl} is the ratio of the sample and R_{stnd} the standard. Standard materials for reference were Vienna Pee Dee Belmnite (PDB) for carbon(C), atmospheric nitrogen (N_2) and SO_2 for sulfur (S).

Principle of compound-specific stable isotope analysis (CSIA for $\delta^{13}C_{AA}$)

We applied CSIA to gain information about the origins of food sources. We focused on essential amino acids (EAA) because consumers cannot synthesize them de novo and therefore depend on them from dietary sources. Metazoans can only synthesize about half of the 20 protein AAs. The EAA can only be synthesized by bacteria, fungi and plants. Organisms that do not synthesize them are obligated to obtain this with their diet.

Algae, bacteria, fungi and plants synthesize essential AA differently (Larsen et al. 2009). These differences in biosynthetic pathways and precursors result in source

characteristic $\delta^{13}C$ patterns among amino acids. Determining the $\delta^{13}C$ composition of those amino acids can therefore provide a distinct signal of the biosynthetic source. This approach is called $\delta^{13}C$ fingerprinting of amino acids and proved to be a valuable tool for identifying food sources to consumers (Larsen et al. 2009, Larsen et al. 2013, Larsen et al. 2015)

Sample Preparation and Analysis for CSIA

For the CSIA in all samples we combined soluble and insoluble material, freeze-dried and homogenized them with a mortar. We weighted at least 2.00 mg ± 0.10 mg of the sample (muscle or otolith), to get a clear signal.

Samples were flushed with N_2 gas, sealed and hydrolyzed in 1-2 ml 6 N HCl (37% HCl diluted with Milli-Q water, Merck, Darmstadt, Germany) at 110°C in a heating block for 20h. After hydrolysis we removed lipophilic compounds by adding 2ml *n*-hexane/DCM (6:5, v/v) to the Pyrex tubes that were flushed shortly with N_2 gas and sealed before vortexing for 30 s. The aqueous phase was then filtered through a Pasteur pipette lined with glass wool that had been pretreated at 450°C. All samples were transferred into the 4ml dram vials before evaporating the samples to dryness under a stream of N_2 gas for 30min at 110°C in a heating block. The samples were stored at -18°C. To volatize the AAs, we followed the derivatization by Corr at al (Corr et al. 2007) methylating the dried samples with acidified methanol and subsequently acetylating them with a mixture of acetic anhydride, triethylamine and acetone (NACME: N-acetyl methyl ester derivatives). To avoid oxidation of amino acids during derivatization, we flushed and sealed reaction vial with N_2 gas. To account for carbon added during derivatization (Silfer et al. 1991) and variability of isotope fractionation during analysis, we also derivatized and analyzed pure amino acids with known $\delta^{13}C$ values. Nor-leucine was used as an internal standard. Amino acid derivatives were injected with an autosampler into an Agilent Single Taper Ultra Inert Liner (#5190-2293) held at 280°C for 2 min.

The compounds were separated on a Thermo TraceGOLD TG-200MS GC column (60m x 0.32mm x 0.25um) installed on an Agilent 6890N gas chromatograph (GC). The oven temperature of the GC started at 50°C and heated at 15°C min^{-1} to 140°C, followed by 3°C min^{-1} to 152°C and held for 4 min, then 10°C min^{-1} to 245°C and held for 10 min, and finally 5°C min^{-1} to 290°C and held for 5 min. The GC was

interfaced with a MAT 253 isotope ratio mass spectrometer (IRMS) via a GC-III combustion (C) interface (Thermo-Finnigan Corporation). From each sample three replicates were made. All this was performed by Thomas Larsen and me in the Leibniz-Labor für Altersbestimmung und Isotopenforschung, Kiel.

Data are expressed in delta values as

$$\delta X = (‰) \; [(R_{smpl}/R_{stnd})-1] * 1000‰,$$

where X is an amino acid, R stand for the ration of heavy to light isotope, where R_{smpl} is the ratio of the sample and R_{stnd} the standard.

We were able to analyze amino acids the following were defined as non-essential for animals: alanine (Ala), asparagine/aspartic acid (Asx), glutamine/glutamic acid (Glx), glycine (Gly), and tyrosine (Tyr). The following were defined as essential: histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), and valine (Val). All amino acid values ($\delta^{13}C$) were corrected for the carbon added during derivatization (Larsen et al. 2013).

Data analysis

We used Excel 2007 (Microsoft Corporation; Redmond, USA) for evaluation of the data. MINITAB (Minitab Incorporated; State College, USA) we used for scatterplots, individual value plots, boxplots and statistical analysis, included paired t-tests, and ANOVAs. We considered the results as significant when $p < 0.05$. To visualize the output of the CSIA results, we performed principal component analysis (PCA) and linear discriminant analysis (LDA).

To assess differences in $\delta^{13}C_{AA}$ patterns between tissue types we applied principal component analysis (PCA) with R version 3.2.2 using the vegan package. PCA is a technique used to emphasize variation and bring out strong patterns in a dataset. PCA achieves this by eliminating uninformative parts. In addition to PCA we examine variables for the ones that visualize the differences for separation of groups the best way by performing LDA. LDA is been applied to emphasizes the differences of groups. We performed LDA with R version 3.2.2 using the MASS package and with MINITAB

Results

Relationship of otolith length / weight and cod individual data

Otoliths grow over the life time of a fish by synthesizing a new growth ring every year, each of those consists of calcium carbonate and protein. To get a better understanding of the growth patterns of Baltic cod's otoliths we studied the otoliths before the preparation for the stable isotope analyses. We measured the length, width and the mass of 12 otoliths and then compared the results with the morphometrics of the equivalent fish. The results show that otoliths grow linearly with the fish i.e., the bigger the fish (TL), the heavier are its otoliths (Fig. 11."I"). The Otoliths length growth is spatially limited, therefore it slows down at a certain size and mass increases (Fig. 11."II"). The otolith's volume increases cubically (a^3) to the length of the fish (Fig. 11."III").
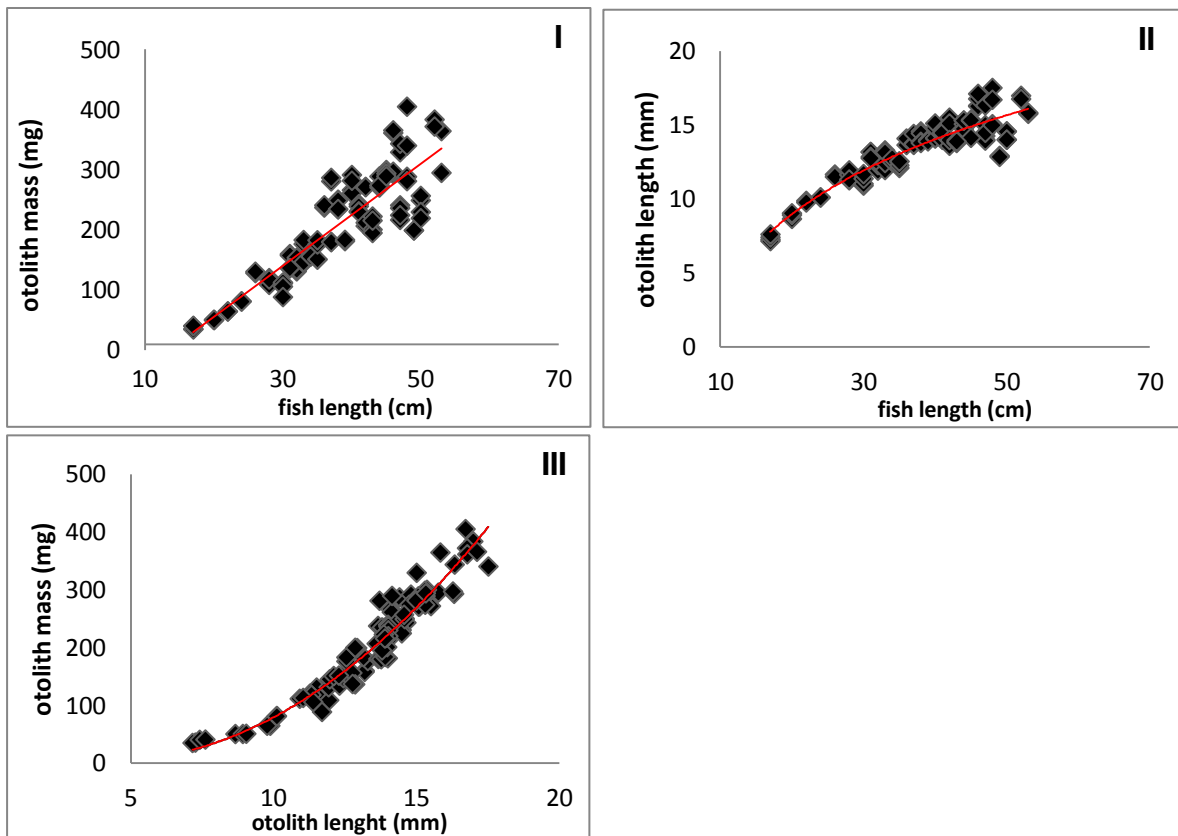

Figure 11. Reults of length, width and mass measurements of the cod otoliths. In figure 'I' the otoliths' mass is plotted in relation to the fish length, the otolith's mass increases proportionally to fish length. Figure 'II' shows the relation between otolith length and fish length.Otolith volume increases cubically to fish length. In figure 'III' the otolith mass is compared to the otolith length.

bulk SIA

Method applicability testing

How do bulk SIA values (C, N, S) differ between muscle tissue and otolith protein of the same cod individual?

In the analysis we compared within 3 groups: otolith-ISOM vs. otolith-SOM (δ^{13}C: -1.6‰ ± 0.4‰; δ^{15}N: 1.9‰ ± 0.3‰; δ^{34}S: 2.4‰ ± 0.8‰), muscle vs. otolith-ISOM (δ^{13}C: 0.4‰ ± 0.3‰; δ^{15}N: 1.6‰ ± 0.3‰; δ^{34}S: 1.7‰ ± 0.6‰), and muscle vs. otolith-SOM (δ^{13}C: -0.3‰ ± 0.3‰; δ^{15}N: 3.5‰ ± 0.3‰; δ^{34}S: 4.1‰ ± 0.8‰). We found significant and consistent differences (paired ANOVA, holm–typ, :p< 0.05) for δ^{15}N and δ^{34}S isotopes in all groups. For δ^{13}C the only significant difference found was between otolith-ISOM and otolith-SOM (Fig. 12, Table 1.).

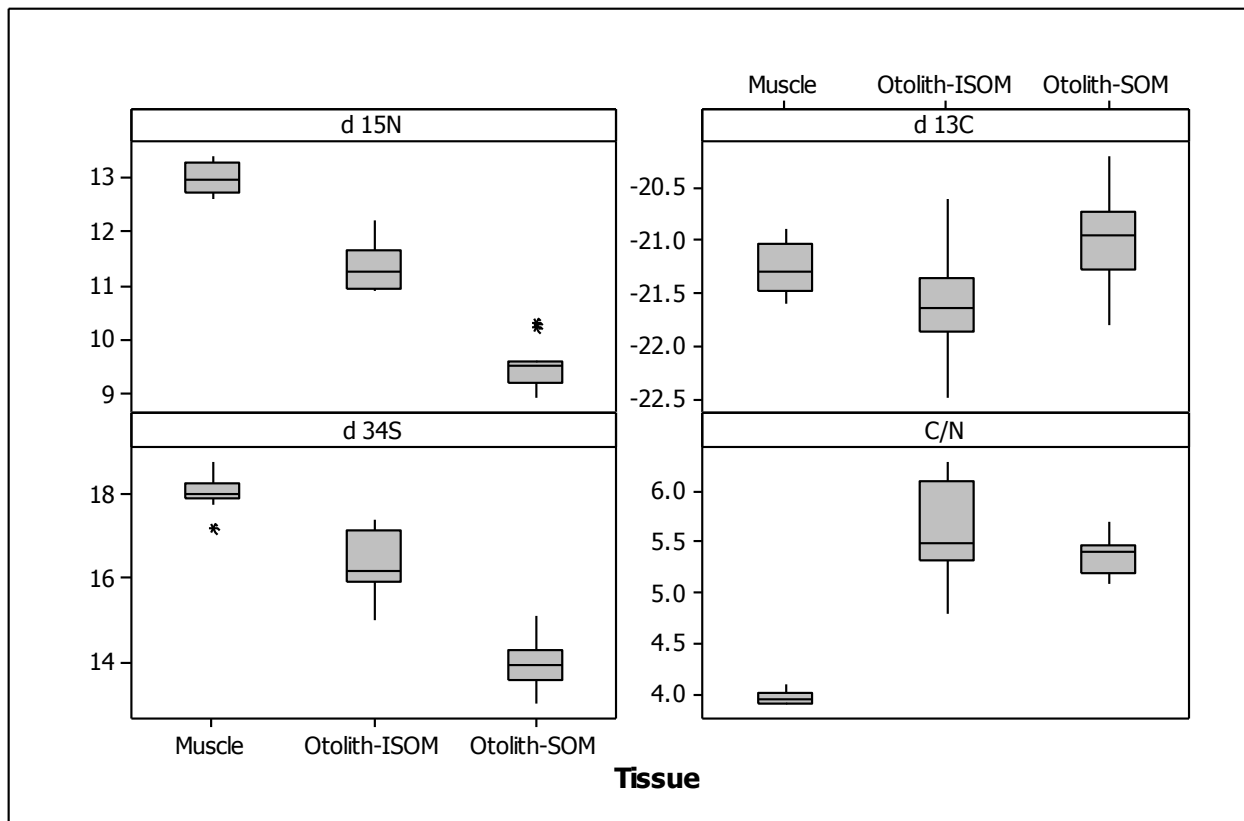**Figure 12. Comparison of δ^{15}N, δ^{13}C, δ^{34}S isotope values of the 3 different sample types visualised in boxplots**. Results <u>before</u> the correction with the tissue-specific fractionation factor, we applied a paired ANOVA (holm-typ)test to verify the comparisons within the isotopes (δ^{15}N, δ^{13}C , δ^{34}S) and the C/N-ratio.

We found that both fractions, ISOM and SOM, lock a comparable diet information like muscle tissue, because of the strong correlation (R > 0.7) that we estimated between individual isotope values of otolith protein and muscle. $\delta^{13}C$ values were almost identical when comparing ISOM with muscle (R = 0.705, p-value = 0.01, R^2 = 49.7) and SOM with muscle (R = 0.758, p-value = 0.004, R^2 = 57.5). ISOM is also linearly correlated to muscle for $\delta^{15}N$ with a relationship of R=0.751(p–value = 0.005, R^2 = 56.5) (Fig. 13.).

Considering both, the consistency of the differences between the analysed sample types and the significant linear regression, we determined shifts (Table 2.). These shifts are a specific difference between a certain otolith protein fraction (ISOM or SOM) and a muscle value. We used the shifts (Table 2.) to provide theoretical muscle values that were in turn used for interpretation in the biological part of this study.

	ISOM vs. SOM			Muscle vs. ISOM			Muscle vs. SOM		
	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S
P-Value	***	***	***	***		***	***		***

Table 1. Comparison of $\delta^{15}N$, $\delta^{13}C$, $\delta^{34}S$ isotope values of the 3 different sample types visualised in a table. The statistical analysis (paired ANOVA) was applied to verify the comparisons. *** p<0,001, ** p<0,01, * p<0,05; red means not significantly different, green means the compared tissues are significantly different.

	ISOM - SOM			Muscle - ISOM			Muscle - SOM		
	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S
Mean:	1.9	-0.6	2.4	1.6	0.4	1.7	3.5	-0.3	4.1
SD:	0.3	0.4	0.8	0.3	0.3	0.6	0.3	0.3	0.8

Table 2. Comparison of $\delta^{15}N$, $\delta^{13}C$, $\delta^{34}S$ isotope values of the 3 different sample types visualised in a table. Here we demonstrate the mean values with the standard deviation of the differences between the isotope values of the sample types.

	ISOM-muscle	SOM-muscle	ISOM-muscle	SOM-muscle
δ15N	R= 0.751	R= 0.603	R-sq= 56.5	R-sq=36.3
p-Value	0.005	0.038	0.005	0.038
δ13C	R= 0.705	R= 0.758	R-sq=49.7	R-sq=57.5
p-Value	0.01	0.004	0.01	0.004
δ34S	R= 0.627	R= -0.138	R-sq=39.3	R-sq=1.9
p-Value	0.029	0.668	0.029	0.668

Table 3. Summary of the linear relationship and regression values of $\delta^{15}N$, $\delta^{13}C$, $\delta^{34}S$ linearly discriminated for ISOM values vs. muscle values and SOM values vs. muscle values. The left column is the output of the linear relationship, the right column is the output of the regression. P-values were estimated for each linare relationship and regression analysis.
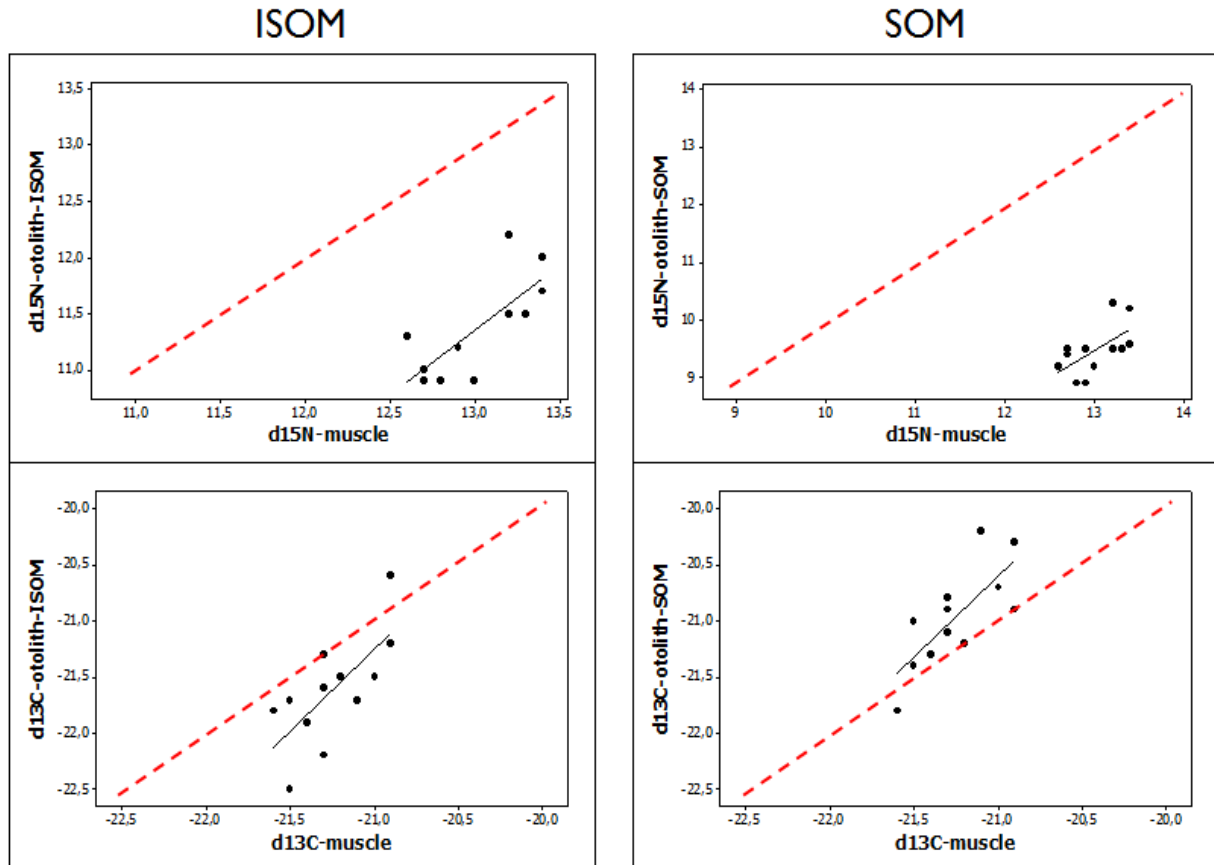
Figure 13. Linear relationship of otolith protein and muscle stable isotope values of Baltic cod. Both otolith fractions, ISOM and SOM were compared with muscle values of the same specimen. Isotope values of δ^{15}N (first row) and δ^{13}C (second row) were plotted. The broken red line represents the 1:1 line. Scales are the same only among the panels with the δ^{13}C values.

How strongly do bulk SIA values differ between otolith protein from left and right otoliths of the same fish?

First of all we compared the morphometrics and performed paired t-tests. We found that otoliths of different sides do not differ significantly in their length (p < 0.903) and weight (p < 0.809). In the literature we could not find any evidence for the similarity of the organic composition between right and left otoliths. To be sure about the similarity of both, we performed bulk SIA on right and left otolith proteins and estimated differences (Table 3.). We compared both organic matrixes: SOM and ISOM values of right and left otoliths (Fig. 14. & 15.).We then analysed the output of bulk SIA with statistical analysis (paired t-test). The results also proved that right and left otoltihs are similar (p > 0.005) in their organic components (Table 4.).

Figure 14. Individual value plots for a comparison between left and right otolith proteins for SOM isotope values (δ^{15}N, δ^{13}C, δ^{34}S) and for the C/N-ratio. We identified no significant difference neither within the isotopes nor the ratio.
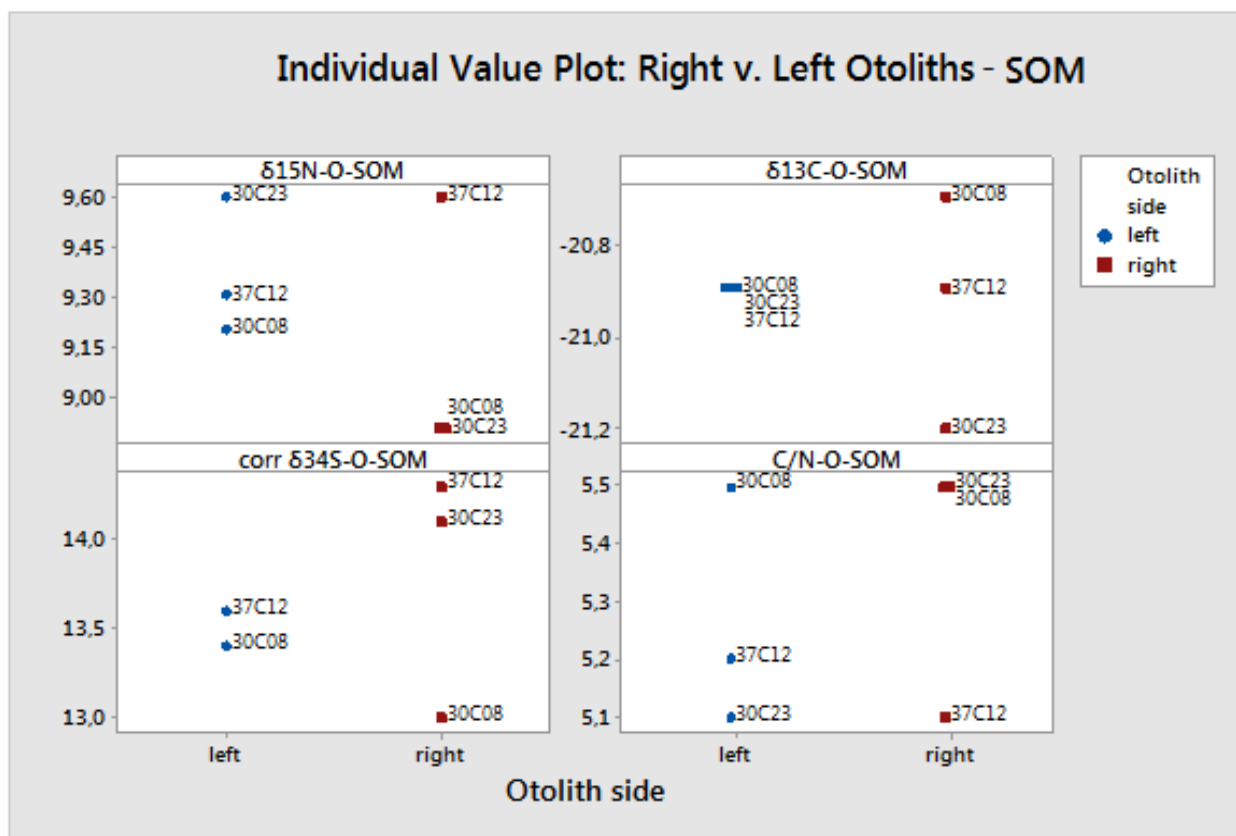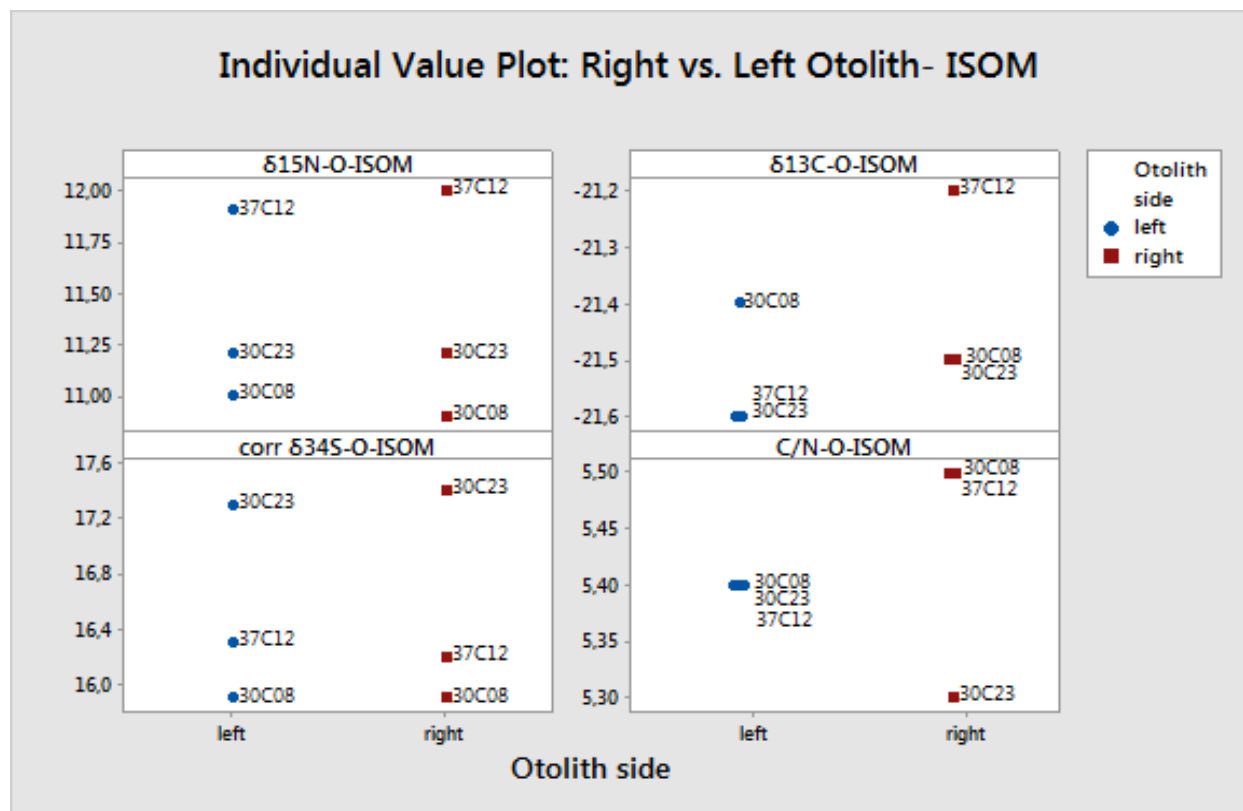

Figure 15. Individual value plots comparing left and right otolith proteins for ISOM isotope values (δ^{15}N, δ^{13}C, δ^{34}S) and for the C/N-ratio. We identified no significant difference neither within the isotopes nor the ratio.

	ISOM			SOM		
	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S
Mean difference	0.0	0.2	0.0	-0.2	0.0	0.1
SD	0.2	0.2	0.1	0.5	0.3	0.7
P-Value	1	0.456	1	0.506	0.84	0.83

Table 4. Summary of results of the paired t-test of bulk SIA values of right and left otolith proteins in order to estimate the similarity of left and right otolith proteins. Our results showed that right and left otolith proteins were not significantly different in their composition (p> 0.005).

Biological case study

Cod in context of prey

We compared the bulk SIA values obtained for cod with values of its potential prey for each of the three analysed delta values (δ^{15}N, δ^{13}C, and δ^{34}S). We first tested whether the application of the tissue-specific fractionation factor reported by Grønkjær et al. 2013 leads to comparable results between the cod otolith SOM fractions and the muscle values of cod (Fig.16.). Since we found no evidence for similarity between these sample types we performed an opportunistic way of interpretation of the bulk SIA. We used the previously estimated shifts to assess the theoretical muscle bulk SIA values for cod's otolith proteins. The calculated theoretical SIA values had comparable signals to the real muscle values of cod, and still differ according to the tissue characteristic variability. We then applied the fractionation factor for muscles (2-4‰) to our theoretical muscle bulk SIA values and interpreted the results considering the potential prey of cod.

Figure 17 and figure 20 show the array of the analysed organisms: the fourbeard rockling (*Enchelyopus cimbrius* L.) had the highest δ^{15}N value of 14.6 ‰, followed by whiting 13.5‰ (*Merlangius merlangus* L.). Both cod- muscle and the theoretical muscle value presented a δ^{15}N mean of 13‰ (Fig. 22.), while flounder had 12.8‰. The invertebrate *Saduria entomon* showed a very similar isotope value like herring with approximately 11.7‰ of δ^{15}N for *Saduria* and 11.6 ‰ for herring. We observed the lowest δ^{15}N value for sprat with 10.5 ‰.

To distinguish between pelagic and benthic feeding fish, we analysed the isotope signatures of δ^{13}C and δ^{34}S.

We observed weak differences between pelagic and benthic organisms by using the δ^{13}C isotope values (Fig. 18) and very clear separation when using δ^{34}S (Fig. 19. & 20.).

According to the figure 18 we identified benthic representatives (the samples with a more enriched δ^{13}C): fourbeard rockling and cod, both with -21.3‰. According to these results the pelagic representatives would be: herring (-22.1‰), sprat (-22.4‰) and *saduria* (-22.4‰). *Saduria* is a well known invertebrate, and it is clearly a benthic representative. Whiting (-21,9‰) and flounder (-22.0‰) were intermediate and therefore no allocation in pelagic or benthic feeding fish could be performed. Thus bulk δ^{13}C analysis proved not to be applicable in our case.

Isotopic signatures of δ^{34}S have proved to be a better choice for distinguishing pelagic and benthic feeders (Fig. 19. & 20.). Here we assumed pelagic feeders to be: herring (17.5‰) and sprat (17.1‰), as well as whiting (19.2‰) and cod (18.1‰).The benthic representatives were determined by their low δ^{34}S isotope values. According to this, benthic feeders were: fourbeard rockling (11.7‰), flounder (11.3‰) and *saduria* (12.4‰).
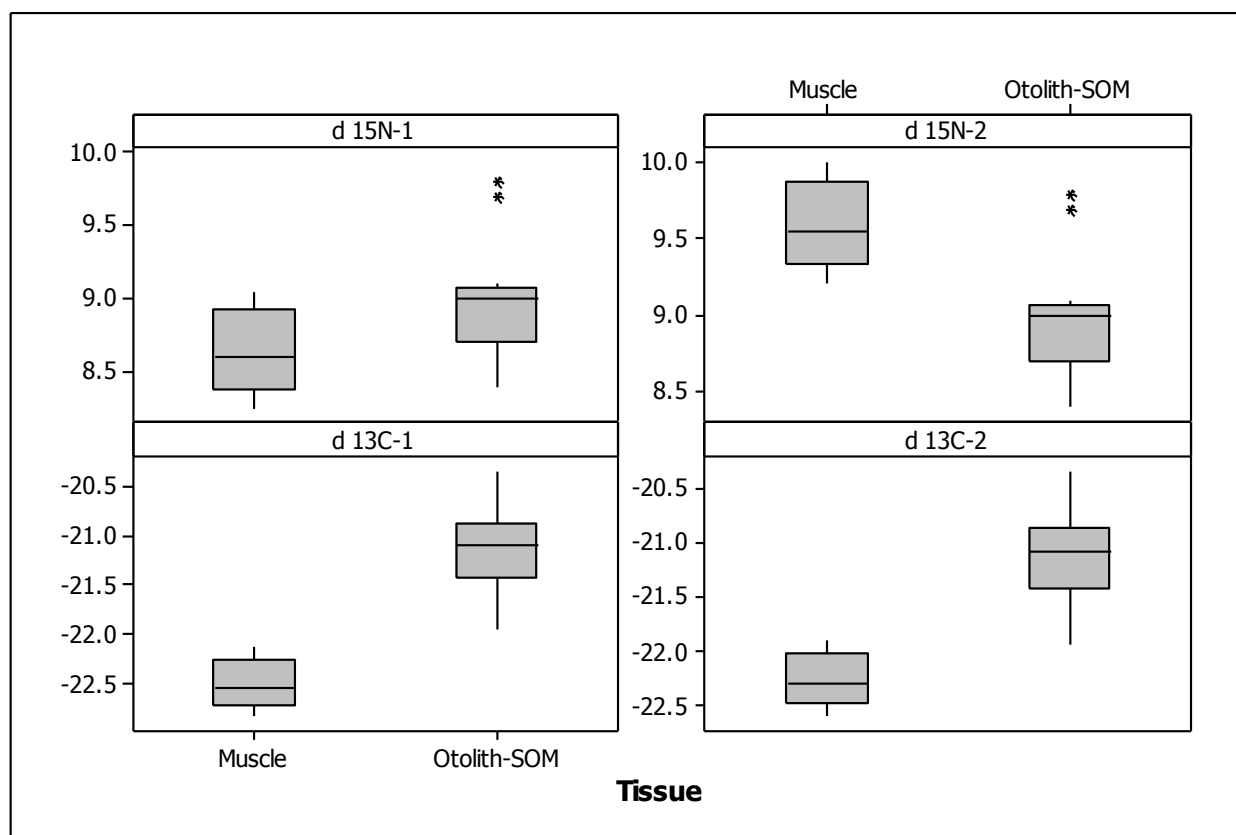
Figure 16. Comparison of δ^{15}N, δ^{13}C, δ^{34}S isotope values of the 3 different sample types visualised in boxplots. Results after the correction with the tissue-specific fractionation factor. The applied t-test showed significant differences between the tissues.

Figure 17. Boxplot of δ^{15}N isotope value of all analysed Baltic-organisms: cod (muscle, otolith-ISOM, otolith-SOM), flounder, fourbeard rockling, herring, saduria, sprat and whiting).
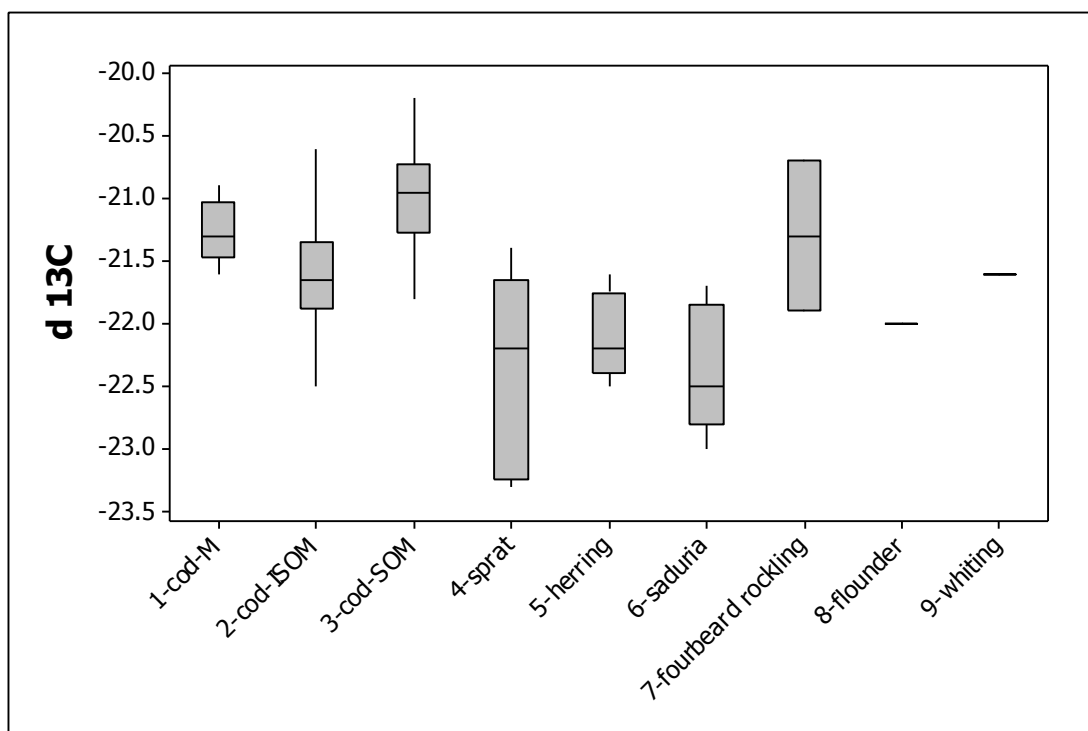
Figure 18. Boxplot of δ^{13}C isotope value of all analysed Baltic-organisms: cod (muscle, otolith-ISOM, otolith-SOM), flounder, fourbeard rockling, herring, saduria, sprat and whiting).

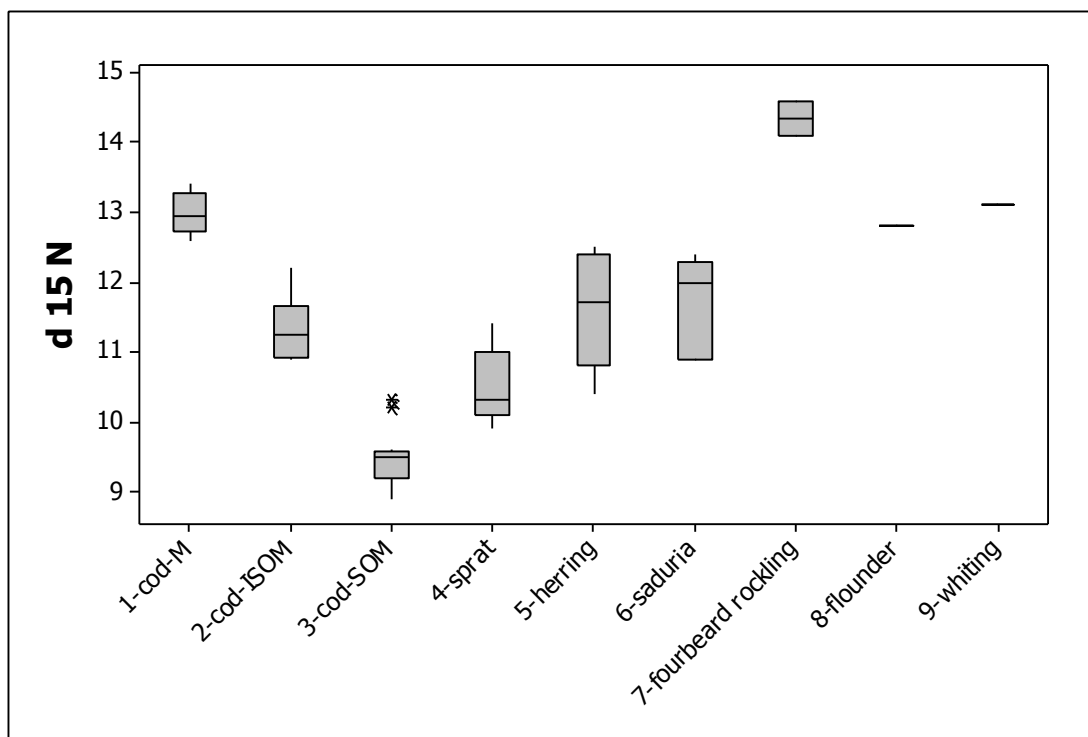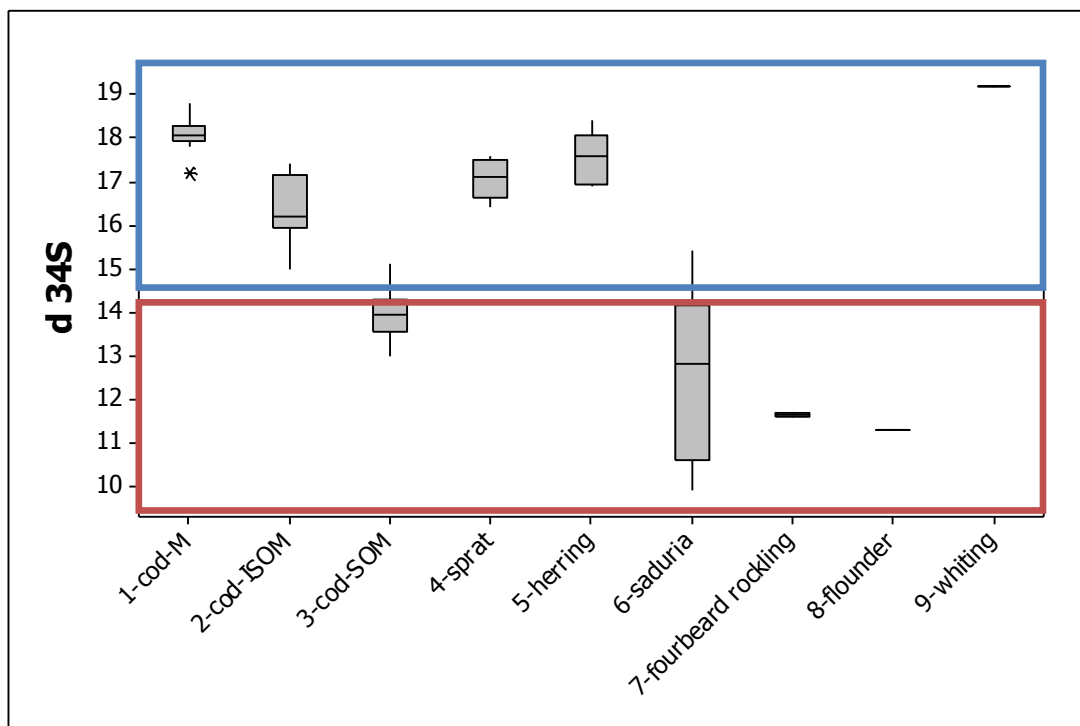**Figure 19. Boxplot of δ^{34}S isotope value of all analysed Baltic-organisms: cod (muscle, otolith-ISOM, otolith-SOM), flounder, fourbeard rockling, herring, saduria, sprat and whiting.** The **Blue** square highlights the species classified as pelagic feeders, the **red** square the benthic feeders.
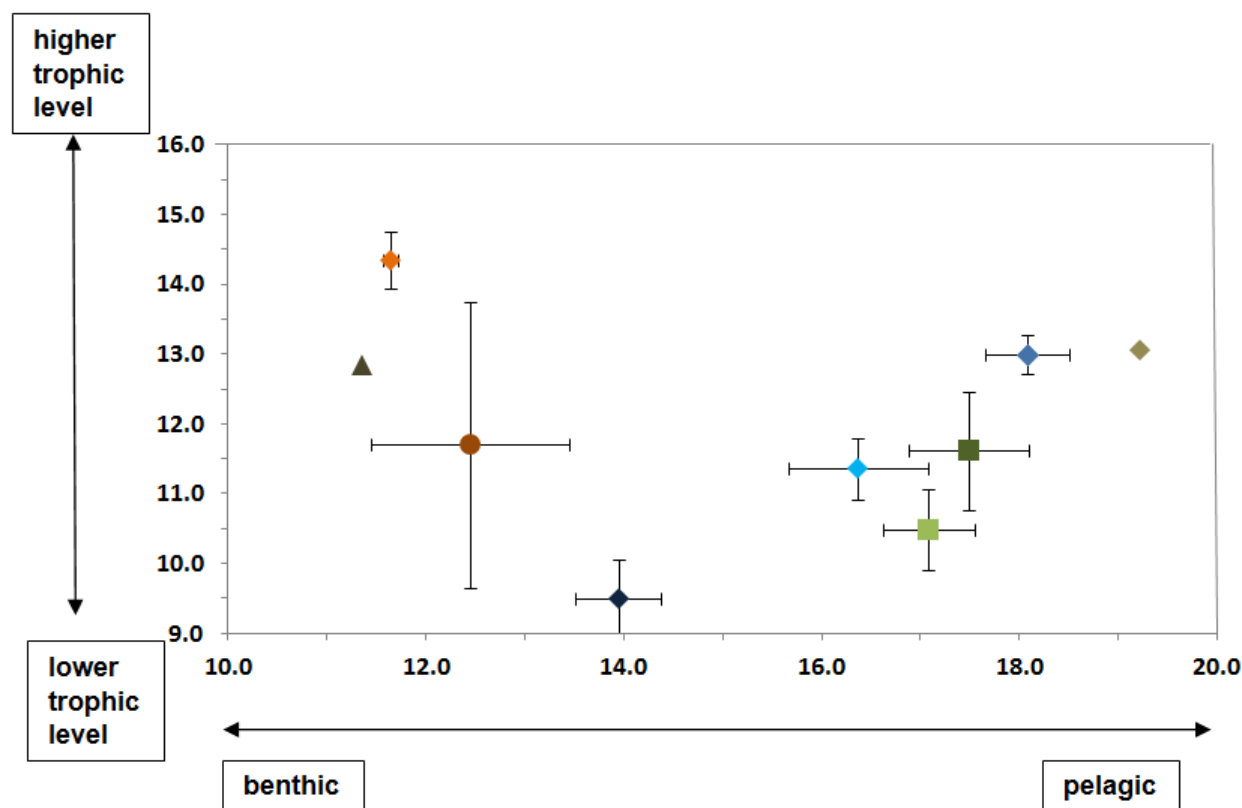
Figure 20. Biplot of mean nitrogen (δ^{15}N) and sulfur (δ^{34}S) values of all analysed species before conversion of the otolith protein values. Standard deviation is presented as error bars. Cod-like fish (rhombus): cod-muscle ( ), cod- ISOM ( ), cod-SOM ( ), whiting ( ), fourbeard rockling ( ); flatfish (triangle): flounder ( ); clupeids (squere): herring ( ), sprat ( ); invertebrate (circle): saduria ( ).

Figure 21. Biplot of mean nitrogen ($\delta^{15}N$) and sulfur ($\delta^{34}S$) values of all analysed species <u>after</u> conversion of the otolith protein values. Here the otolith SOM fraction was converted into the theoretical muscle values. After the conversion we applicated the tissue-specific enrichment factors of muscles on the cod data. The red arrows present the correction for the 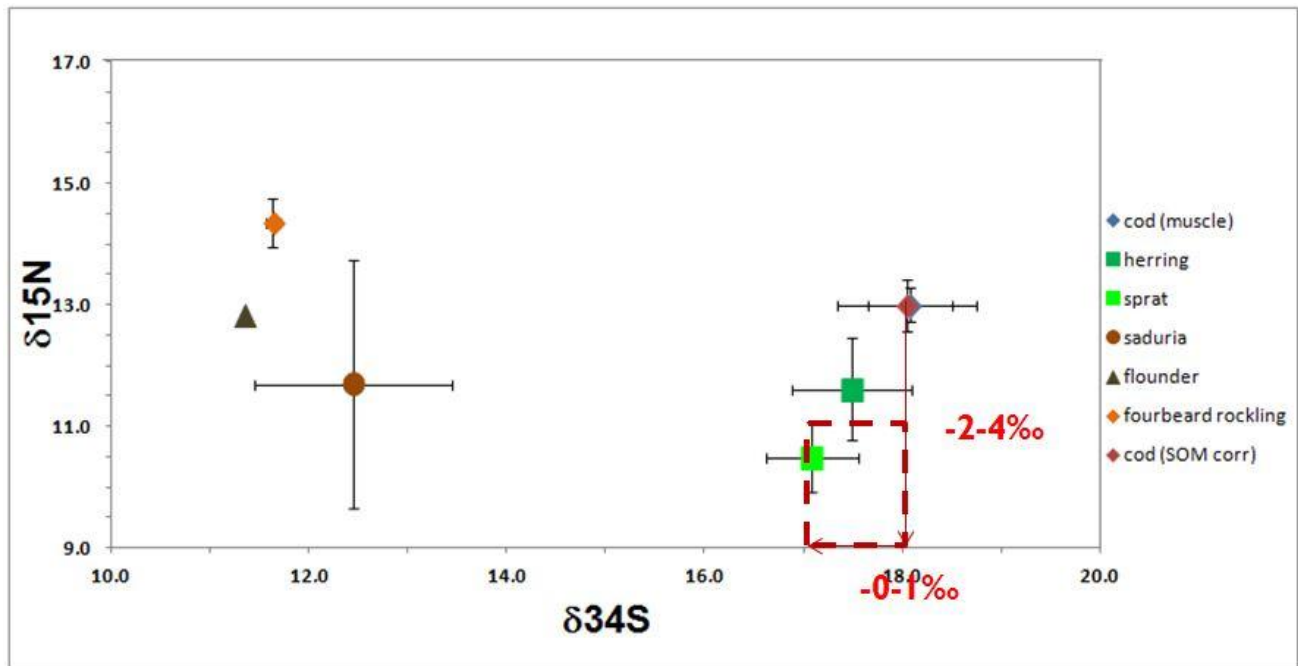enrichemnt factors. The drawn square presents the isotopic area of cods prey. Sprat's isotope values fit almost completely into the square, indicating sprat to be the main food source of cod. Standard deviation is presented as error bars.

How strongly do cod individuals differ in isotopic values?

	Muscle			Otolith ISOM			Otolith SOM		
	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S	δ15N	δ13C	δ34S
Mean for all cod	13,0	-21,3	18,1	11,4	-21,6	16,4	9,5	-21,0	13,9
sd for all cod	0,3	0,2	0,4	0,4	0,5	0,7	0,4	0,5	0,6
Mean fo BIG	12,9	-21,5	18,3	11,0	-21,8	17,0	9,4	-21,4	13,7
sd for BIG	0,2	0,1	0,5	0,2	0,1	0,4	0,2	0,4	0,4
Mean fo MIDDLE	13,0	-21,1	18,1	11,5	-21,3	16,2	9,5	-20,8	13,9
sd for MIDDLE	0,3	0,2	0,3	0,5	0,4	0,6	0,5	0,3	0,7
Mean fo SMALL	13,1	-21,3	17,8	11,5	-22,1	16,0	9,6	-20,8	14,2
sd for SMALL	0,4	0,2	0,6	0,2	0,4	1,0	0,5	0,6	0,5

Table 5. Summary of mean isotope values and the standard deviation of the bulk stable isotope analysis (bulk SIA) assessed for each cod- size class (BIG, MIDDLE, SMALL)and for each fraction (muscle, otolith-ISOM, otolith-SOM**).**

In bulk SIA the mean values were: for muscle: $\delta^{15}N$: 13.0‰, $\delta^{13}C$: -21.3‰, $\delta^{34}S$: 18.1‰, for otolith-ISOM: $\delta^{15}N$: 11.4‰, $\delta^{13}C$: -21.6, $\delta^{34}S$: 16.4‰, otolith-SOM: $\delta^{15}N$: 9.5‰, $\delta^{13}C$:-21.0‰, $\delta^{34}S$: 13.9‰.

We observed low variation (small standard deviation) in all cod-size groups for δ^{15}N. Within the specific cod size rages (cod length: BIG: 50-55cm, MIDDLE: 40-45 cm, SMALL: 35-39 cm) we observed little to almost no differences in the mean values of isotopes (Table 5.). In SMALL cod we observed the highest variation (high sd value) in all sample types and for all isotopes.

We identified no correlation between δ^{34}S and the TL of the analysed cod, which means no benthic-pelagic shift in cod (Fig.22.)
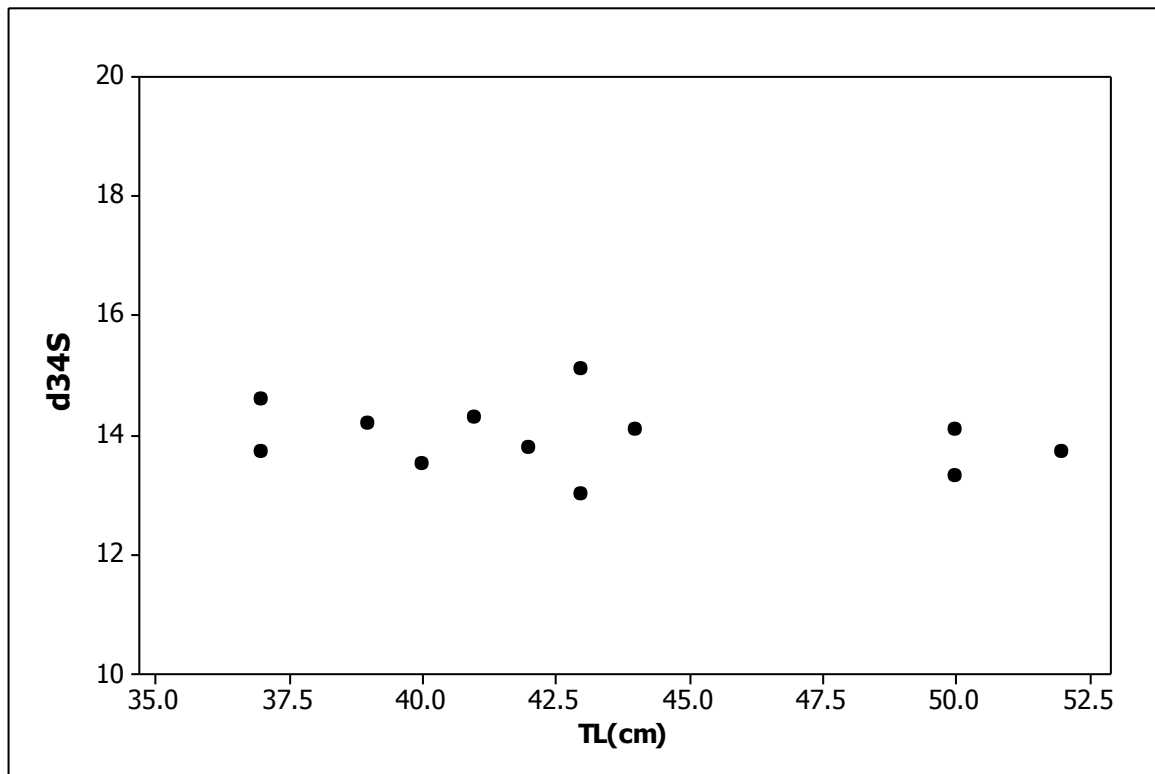
Figure 22. Diagram to visualise the lack of an ontogenetic shift within the analysed cod samples. We estimated that cod sampled 2014 do not follow the benthic pelagic shift. The cod in total is an almost exlusiv pelagic feeder.

CSIA

Method applicability testing

How do CSIA (C) values differ between muscle tissue and otolith protein?

The PCA with non-normalized δ^{13}C$_{AA}$ values of Ile, Leu, Lys, Phe Tyr, Val showed that most of the variability can be explained by differing isotope baseline values rather than isotopic variability between individual EAA (Fig. 22.).The first axis (PC1)

explains 87.5% of the variability, whereas the second axis (PC2), explains 5.7% within our data set.

According to figure 22 almost all EAA were important for separating the groups within our data set. Both muscle and otolith protein yield comparable results, this allowed to group them for the following analyses.
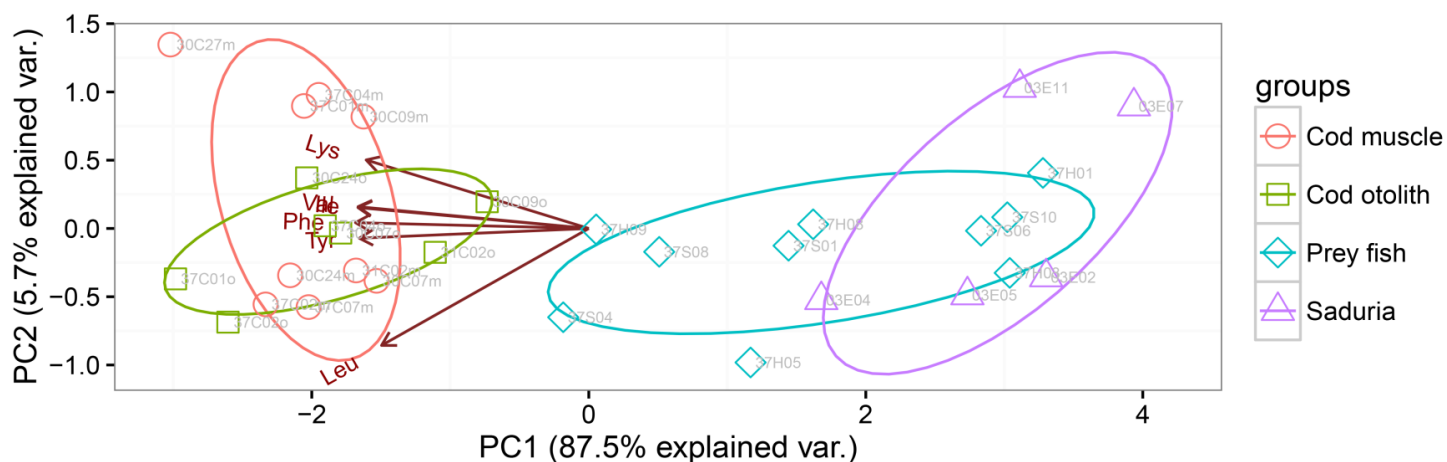
Figure 23. Principal component analysis of δC_{AA} patterns of different organisms of the Baltic Sea (saduria, prey fish like herring and sprat, cod) and different tissues (cod-muscle and cod-otoliths). First axis (PC1) explains 87.5% of the variabiliy in our offset, whereas the second axis (PC2) explains only 5.7%. All the analysed samples are clustert to the predefined groups.

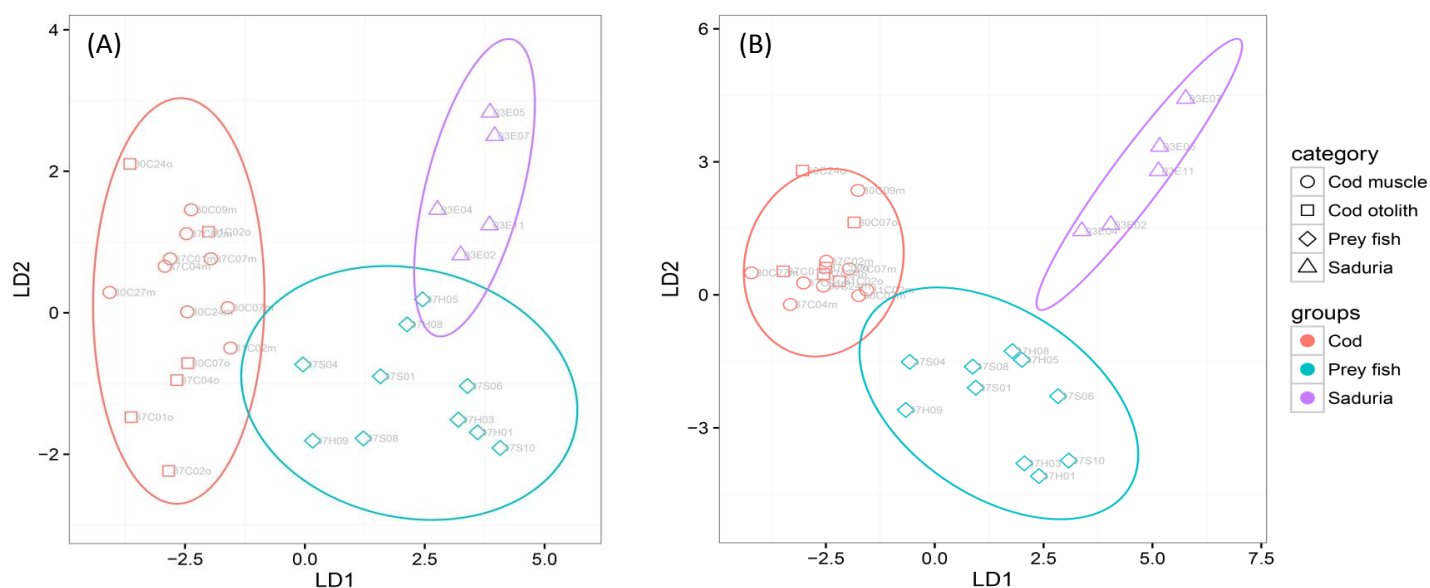**Figure 24. Linear discriminant analysis based on the $\delta^{13}C_{AA}$ patterns of different organisms of the Baltic Sea (saduria, prey fish like herring and sprat, cod) and different tissues (cod-muscle and cod-otoliths).** The $\delta^{13}C_{AA}$ values of Phe, Val, Lys, Ile influence most in both LDAs. In LDA 'A' we used only AA. All analysed spezies are clustered into groups.

Cod in context of prey

After PCA, we performed LDA on $\delta^{13}C_{EAA}$ data to create a classification model with cod, prey fish (herring and sprat) and *Saduria*. LDA is also useful to assess the EAA that are most informative. Hence it provides a tool to discriminate between groups and to assess overall differences and similarities in $\delta^{13}C_{EAA}$ patterns among these three predefined groups. As the results of the PCA revealed similar $^{13}C_{EAA}$ patterns for cod muscles and otolith proteins we grouped these together in the LDAs (Fig.24.).

We performed two LDAs: in the first one (Fig.24. 'A') we only included $\delta^{13}C_{EAA}$ values and in the second one (Fig.24.'B') we also included $\delta^{34}S$ isotope values to examine a possible increase in the discriminatory power of the LDA. In the first LDA (Fig.24.'A') the most important EAA for distinguishing between cod and their potential prey were Phe, Val, Lys, Ile. In figure 24 (A), the EAA with the greatest absolute linear discriminant values for the first axis were Phe (-0.84) and Val (0.33). For the second axis, the two most informative EAA were Lys (1.86) and Ile (-1.88). Plotting of these AAs reflected the best discrimination between cod's sample types and the analysed potential prey.

We determined the patterns for the benthic-pelagic affinity ($\delta^{34}S$ isotope values) of the analysed organisms which can be used to examine cod's feeding ecology. By adding $\delta^{34}S$ isotope values to the LDA model (Fig.24.'B'), the three groups became more distinct. Notably, all the cod samples clustered more closely. For LDA (Fig.24.'B') also Phe, Val, Lys and Ile had the greatest impact for distinguishing the analysed sample groups. LDA (Fig.24.'B') showed a clear difference between pelagic and benthic prey in the $\delta^{13}C$ E_{AA} values. We observed a distinction between Saduria's benthic isotope signature and the pelagic isotope signature of the prey fish. Even if cod's signature is separated from both these groups, it shows a slight affinity towards the pelagic prey values.

Discussion

In this study we confirmed the organic matrix of cod-otoliths to be a good tool for a temporal reconstruction of its past feeding habits. Especially promising is the bulk stable isotope analysis (C,N,S), where a comparison between otolith protein fractions and the traditionally used muscle tissue showed systematic and highly predictable shifts. These allowed the conversion of otolith protein bulk SIA values to theoretical muscle values. This is essential as for the latter an interpretation in food web studies is already established (Fig. 21.). Additionally, we confirmed the similarity between bulk SIA results for the left and right organic matrix of otoliths. Therefore in further studies only one otolith has to be used for analysis, saving the other one for possible additional analyses such as microchemistry analysis.

This study is one of the first that applied amino acid specific SIA on fish otolith protein for a commercially important fish. The comparable values of otolith proteins and muscles confirmed the high potential of their use in studies where muscle tissue is impossible to obtain. CSIA showed similar amino-acid isotope values for otolith proteins and muscles. This confirmed the results of the bulk SIA.

We gained a further insight into the diet of cod by including bulk isotope values into the framework of its potential prey. The results of the converted otolith protein did not show a benthic-pelagic shift, and were consistent with an almost exclusively pelagic diet signal. The results and assumptions of the bulk SIA were confirmed by the CSIA and are also comparable to previous muscle bulk SIA analysis (Mohm & Dierking *unpublished*).

Otolith protein bulk and compound-specific SIA method feasibility

Otoliths and muscles are located in different parts of a fish's body and synthesized therefore in different synthesis pathways. Even their compounds, like the otolith-protein matrix, are not homogeneous. It consists of water-insoluble and water-soluble organic matrix both having different properties due to their different biochemical compositions (Hüssy et al. 2004). A previous SIA study of Grønkjær et al. 2013 showed that both otolith fractions, ISOM and SOM, are significantly different in their isotope signature. Grønkjær et al.2013 suggest that the distinct amino acid composition of

both results in a different routing and fractionation of the amino acids within otolith proteins thus causing the different isotope values. Additionally to the distinct ISOM and SOM isotope signatures we observed a significant difference between these otolith protein fractions and muscles. We concurred with Grønkjær et al.2013 and thus attributed this variation in isotope signatures to a comparable routing and franctionation difference of amino acids.

Usually tissue-specific fractionation factors are used for correction of the raw delta values to establish food web linkages. Grønkjær et al.2013 presented in their work the tissue-specific fractionation factor for otolith-SOM (0.51‰ for δ^{15}N and 0.14‰ for δ^{13}C) and for cod muscles(4.36‰ for δ^{15}N and 1.23‰ for δ^{13}C). By applying the tissue-specific enrichment factors to our muscle and SOM samples for both delta values (δ^{15}N and δ^{13}C) we still noted significant differences. Although after the assessment of the fractionation factors, both should have given similar signals. As the SOM fractionation factors have only been calculated once using juvenile cod samples Grønkjær et al.2013 addressed the importance of gaining a more profound understanding of these factors especially for older cod specimen as their different metabolic turnover could have an impact on isotope routing. All in all we assumed, that the tissue-specific fractionation factor estimated by Grønkjær et al.2013 could thus not be applied to our data.

Using calculated shifts between the bulk SIA values of muscles and otolith protein fractions proved to be a good solution enabling an interpretation of the isotope signature of otolith proteins even though an otolith-specific fractionation factor was not available. The predictability and the small variation of the shifts allowed for a reliable convertion of otolith protein bulk SIA values to theoretical muscle bulk SIA values. The estimated shifts for example: approx. 1.6‰ ± 0.3 for δ^{15}N and 1.7 ‰ ± 0.6 for δ^{34}S between muscle and ISOM values (Table 2.) were added to the otolith ISOM fraction values. This resulted in theoretical muscle values, which could be used for further analysis.

The reliability of the shifts is also enforced the by the recorded strong linear relationship: the correlation between δ^{13}C bulk values of otolith proteins and muscles, as well as between δ^{15}N bulk values of the otolith-ISOM fraction and the muscle. This positive output of the correlation underlines that the record of dietary information in otoliths and muscle is almost equal (McMahon et al. 2011).

If, according to the linear relationship, the record of diet is the same in both structures why do we still have differences in bulk SIA between the otolith proteins and muscles?

The bulk SIA reflects isotope values from both: EAAs and non EAAs, because it analyses whole structures. Previous studies report, that non EAAs fractionate much stronger whereas EAA undergo almost no fractionation. Therefore, tracing of the EAA is a good tool for feeding ecology studies (McMahon et al. 2010). The differences we noticed between bulk SIA otolith protein values and bulk SIA muscle values can be attributed to the fractionation of non EAAs. We performed CSIA to confirm this and to gain higher resolution isotope values of otolith proteins and muscles. This enabled a better benthic – pelagic distinction of the Baltic cod's diet. CSIA is a method that first separates all the AAs using gas chromatography and then analyses the isotope values of each AA separately (Larsen et al. 2009, Larsen et al. 2013). We then concentrated on the isotope values of the EAA of muscles and otolith proteins to track cod's feeding-target groups. We performed PCA to best compare single otolith $\delta^{13}C_{EAA}$ signature with the muscle $\delta^{13}C_{EAA}$ signature of the same specimen (Fig. 23.). After the application, otoliths and muscles values presented comparable results. Both were clustered into one group, separated almost completely from other analysed organisms. In contrast to bulk SIA (which is influenced by both non –EAA and EAA) CSIA provided clear evidence that the changes in otolith proteins and muscle can be related to the fractionated non EAA

In the PCA we focused on the isotope signature of EAAs. Those are the most important components for multi cellular organisms, like fish. EAAs are indispensable for many biosynthetic pathways as metabolism and growth. In the water column Microorganisms and algae (phytoplankton) synthesize half of the AA, especially the EAA, which multi cellular organisms are obligated to obtain from their diet. EAA proved to do not undergo fractionation within calcareous structures while passing by, therefore they show direct connections between EAA-producers and their predators (O'Brien et al. 2002, McMahon et al. 2010). In our case the EAAs isotope signatures of muscles and otoliths can be used to distinguish between the benthic and pelagic origin of cod's prey.

Our results confirmed the assumptions made in other studies about the similarity of EAA isotope values of otolith proteins and muscles (McMahon et al. 2011).

To investigate the benthic – pelagic diet of cod, we applied LDA (Fig.24.'A') on our data. To improve the LDA we added $\delta^{34}S$ isotope values to the model (Fig.24.'B'), which resulted in more distinct clusters of the analysed samples. Notably, all cod samples grouped more closely. LDA (Fig.24.'B') shows a clear difference between pelagic and benthic prey in the $\delta^{13}C$ E_{AA} values. We distinguish between Saduria's benthic isotope signature and the pelagic isotope signature from prey fish (herring and sprat). Even if cod's signature is separated from both these groups, it shows a slight affinity towards the pelagic prey's values (as can be seen in the small overlap in LDA (Fig. 24. 'B') between the cod and pelagic-prey group). Nevertheless, a definite relationship can still not be made. For a better estimation of the relationship, it would be particularly important to extend the groups of possible prey for cod, by addressing more species. We assume that due to the ongoing ecosystem changes in the Baltic Sea, having a big impact on cod as its top predator, we could have missed a major food source in our analysis - from what we know about the well known ecosystem (Eero et al. 2015), this is not very likely though.

Another explanation for the small overlap might be the geographic origin of the prey (herring and sprat) group. Both herring and sprat are migratory fishes (Aro 1989) and might thus feed in a geographic area outside the Bornholm Basin only migrating there prior to or during sampling. Different area can evoke a different isotope baseline(Mohm & Dierking *unpublished*), while feeding in several areas can lead to strongly varying isotope values, hardly to interpret.

Even though it is not very likely that EAA would undergo routing by passing thru the food web (Rowell et al. 2010) this could lead to different values in different structures of the fish body compared to source material and therefore unclearness in defining cod's food source.

Comparison of left and right otolith protein bulk SIA

If bulk SIA results from left and right otolith proteins of the same individual are consistent, one otolith of each pair could be saved for additional analyses in future studies, e.g., genetic or microchemistry analysis, without negatively affecting the quality of SIA results. Previous studies only assessed similarities in morphometrics (weight, length) between otoliths of the same fish (Hunt 1992). No information about the simi-

larity of bulk SIA values of otolith protein from left and right otoliths was available. In our study we confirmed similarity of bulk SIA values of left and right otolith, therefore similarity of their composition. This finding allowed us to split the otoliths in this study. Right otoliths were used for bulk analysis (bulk SIA) and left for the compound-specific approach (CSIA). The conclusions drawn from our results are important regarding future purposes, like the retroactive reconstruction of ecological time series from stored otoliths. Otoliths stored in museums are valuable in historical terms and should be used very carefully. The estimated similarity of both otoliths allows sacrificing one and saving the other one for future studies.

Assessment of benthic vs. pelagic feeding of cod

The benthic – pelagic feeding of Baltic cod sampled in 2014 was analysed by comparing the bulk isotope signature of cod's theoretical muscle values obtained from otoliths with the bulk isotope signature of its potential prey. We estimated cod to be the top predator fish of the Baltic sea, with a high δ^{15}N (13.0‰) value compared to the other analysed fish. A largely pelagic diet of cod was indicated when we compared benthic representatives (*Saduria*, fourbeard rockling and flounder) and pelagic ones (herring and sprat). This was particularly evident when considering δ^{34}S values. It seems that the decrease of the benthic organisms abundance like *Saduria* and other crustaceans last years (Carstensen et al. 2014) affects cod's diet.

Traditionally δ^{13}C values are used for the benthic pelagic distinction. Concerning the allocation of *Saduria*, whiting and flounder this approach proved to be inappropriate in our study. We therefore advise δ^{13}C values for benthic-pelagic differentiations to be interpreted with caution for organism from the Baltic Sea. As opposed to this, the δ^{34}S values distinguished benthic and pelagic organisms of the Baltic Sea very clearly. We confirmed δ^{34}S values to be the better, more reliable choice for this study.

Within cod no clear signal of an ontogenetic shift, e.g., based on δ^{34}S, was visible. However, the size range of organisms was limited by the CSIA analysis: only cod > 35 cm could be analysed. It may be particularly interesting to analyse smaller individuals (< 35 cm) while considering a diet switch within cod of different sizes.

Herring and *Saduria* are very different in their occurring and feeding locations according to the δ^{34}S. S*aduria,* with a low δ^{34}S, is living and preying on benthos while herring is a pelagic fish and has therefore a high δ^{34}S isotope value. Thus it is surprising that *Saduria entomon* and herring have very similar δ^{15}N isotope values, which perhaps do not indicate the same trophic level. Herring feeds on zooplankton (Haahtela 1990, Möllmann et al. 2004), and is a secondary consumer preying occasionally on nektobenthic prey too. *Saduria's* diet can be very mixed, consisting of all kinds of dead animals, including fish (Haahtela 1990). The similarity in δ^{15}N for both of the organisms can be explained by these differing δ^{15}N baselines of their habitats.

The overall benthic - pelagic results of cod's diet can be partially supported by the results of the CSIA. Here we confirm the assumption of cod's increased pelagic predatory behavior, through the affinity of cod's isotope values towards the pelagic isotope signatures.

Caveats, lessons and next steps (bulk SIA and CSIA)

SIA measurements of otoliths provide reliable results when considering the specific shift between otolith and muscle delta values. With the applied extraction method for otoliths we receive two protein fractions that can both be used for further SIA analysis. From our experience with handling both fractions we prefer the ISOM fraction, which (returned in form of a pellet) is easier to handle and use than SOM. In future studies we should therefore investigate more afford for a better assessment of the ISOM fractionation.

This is one of the first studies that performed CSIA on a commercially important fish like cod. CSIA showed the important patterns of distinction of the analysed Baltic community representatives and at least in part confirmed the bulk SIA conclusions. Nevertheless it left some uncertainty. In further work it would be needed to integrate spatial differences in prey values to exclude the possible isotope signal of migrated fishes. It is also important to extend the prey pool of cod for a well defined estimation of its trophic relationships. Also a more extended study with more individuals would be more informative concerning the diet of Baltic cod. Using the consistent relationship between otolith length and weight and the fish length the minimal fish length needed to obtain otoliths heavy enough to perform CSIA (2mg) could be

estimated. It would be particularly helpful to adjust the method for smaller sample individual sizes. Including the analysis of smaller individuals, could give more profound conclusions about the benthic-pelagic ontogenetic shift.

Approach towards long-term reconstruction of cod's benthic vs. pelagic feeding

The study presents a tool for feeding ecology reconstruction out of otolith protein bulk SIA values. We identified systematical shifts between the otoliths (SOM or ISOM) and muscles that allow further analysis referring to cod's potential prey. Our data contributes to more knowledge in the general use of the bulk SIA and the CSIA. The knowledge about the interpretation of isotope values for hard materials like otoliths opens new possibilities for ecological studies.

It has been considered that one of the predominant reasons for cod's bad condition nowadays is the decreasing benthic food availability reflected in the change of the cod's diet (Carstensen et al. 2014, Eero et al. 2015). Our results for the cod from 2014 showed an almost exclusive pelagic feeding behavior, while conclusions about the diet change from former years could not be drawn so far. To provide reliable information about the change of cod's diet since 1980, more investigation on this topic is definitely needed. For example the application of the bulk SIA to the otoliths from the 30 years collection, which would be a pioneering study as such, providing a retrospective long-term data series from otolith proteins. This long-term data series would contribute to a more extensive knowledge about cod's diet and its feeding preferences of the past years. Thus it might be a crucial tool to answer questions concerning the ongoing bad condition of cod. Furthermore questions related to foodweb changes within the Baltic Sea and the impact of climate change on this brackish ecosystem could be answered by using this retrospective data-set.

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Acknowledgments

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Die eingereichte schriftliche Fassung der Arbeit entspricht der auf dem elektronischen Speichermedium.

Weiterhin versichere ich, dass diese Arbeit noch nicht als Abschlussarbeit an anderer Stelle vorgelegen hat.

Ort, Datum   Unterschrift