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Discrimination potential of otolith chemistry to distinguish two parapatric species of flounder (*Platichthys*) in the Baltic Sea

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ABSTRACT

Baltic Sea flounder were recently split into two species, the offshore spawner *Platichthys flesus* and coastal spawner *Platichthys solemdali*. The two species can only be distinguished based on egg and sperm characteristics and via genetic analyses, which limits the species identification methods of larvae and juveniles to molecular techniques. We investigated whether otolith chemistry could be used as an additional tool to identify flounder to species level. We tested for species-specific differences in otolith multi-elemental signatures and spatial consistency of those differences for the early life stages of flounder in three areas of the central Baltic Sea (ICES SD 24–28), where the distribution of both species overlaps. Otolith chemistry signatures obtained through maternal transfer (i.e. core chemistry) and signatures that reflect the post-hatching phase were not significantly different between species. Species-specific differences at the sub-regional scale were only found for the Latvian coastal survey area for multiple elements (Ba, Cu, Mg, Pb, Sr and Zn), but were insufficiently distinct for reliable species discrimination. Geographic classification of age-0 juveniles to survey area was more successful than classification to species, which was reflected by a spatial trend in otolith Sr:Ca that followed the salinity gradient and higher Mn:Ca and I:Ca for Latvian individuals. Otolith chemistry of early life flounder from the Baltic Sea reflects spatial variability in environmental conditions but does not differentiate between the two flounder species in sympatric habitats.

1. Introduction

Steep abiotic environmental gradients can restrict aquatic ecological communities to species with adaptations that will allow them to cope with stressful conditions (Attrill and Rundle, 2002; Basset et al., 2013; Reizopoulou et al., 2014). The Baltic Sea is the largest brackish inland sea in the world and is characterized by a pronounced salinity gradient (Bagge, 1981; Ehlin, 1981; Leppäranta and Myrberg, 2009). The intrusion of saline water into the Baltic Sea (ICES subdivisions (SD) 24–32; Fig. 1) is restricted by a network of deep and narrow channels and shallow sills at the entrance area, the Danish Straits (SD 22–23).

In addition, the Baltic Sea has a large catchment area at about four times the areal extent of the sea itself, resulting in a positive freshwater budget and net outflow conditions (Elken and Matthäus, 2008). These hydrological conditions create a steep salinity gradient (using the Practical Salinity Scale), from about 16–20 at the beginning of the Danish Straits to 8–10 before water enters the main basin (SD 24–32), to low salinities of 2–3 at the northern and far eastern end (Furman et al., 2014; Leppäranta and Myrberg, 2009). The outflow of low-salinity surface water and the inflow of higher-salinity water along the bottom layers lead to permanent vertical stratification in the central sub-basins (SD 25–29), which prevents ventilation and leads to hypoxic and even anoxic conditions in these areas (Meier et al., 2006; Omstedt et al., 2014). The large spatial and temporal variations in salinity and oxygen concentrations of the Baltic Sea sub-basins restrict colonization of marine biota from the North Sea to species that have wide environmental

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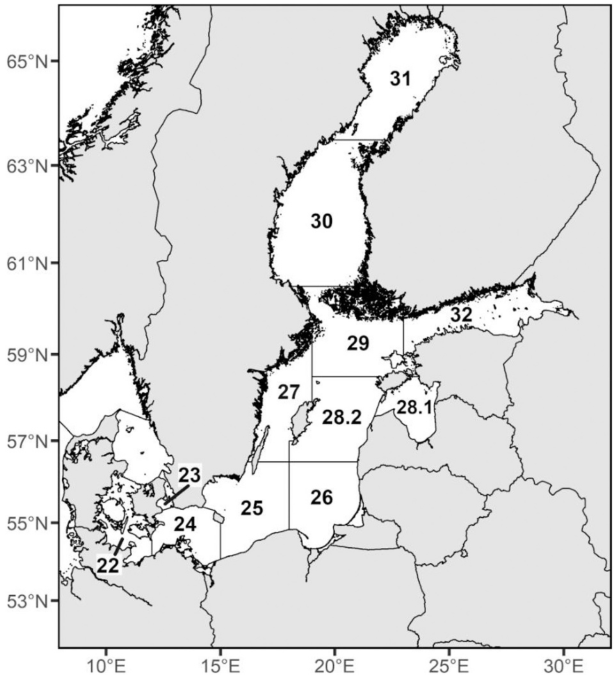

Fig. 1. Baltic Sea and transition area to North Sea with ICES subdivisions (SD) referred in the text.

tolerance ranges (Ojaveer et al., 2010; Snoeijs-Leijonmalm et al., 2017).

The European flounder *Platichthys flesus* (Linnaeus, 1758) is one of the few fish species that has been able to establish populations in the Baltic Sea (Florin and Höglund, 2008). It is a highly euryhaline species that can live in water of salinities ranging from 0 to 35 (Lundgreen et al., 2008; Waede, 1954). Adult flounder can be considered as facultative catadromous and migrate into estuarine waters to spawn (Daverat et al., 2012). Larvae and juveniles have even been found to migrate into riverine freshwater habitats throughout Europe and move back to the brackish areas of the estuary at a later stage to continue their growth and development (Daverat et al., 2011; Dias et al., 2017; Freitas et al., 2009; Kerstan, 1991; Summers, 1979). Juvenile flounder exhibit plasticity in the timing of movements across salinity zones along the estuarine gradient and in the duration of residence within a salinity zone, with some individuals undertaking multiple migrations between the brackish estuarine habitats and freshwater habitats (Daverat et al., 2011, 2012; Dias et al., 2020). Flounder in the Baltic Sea undergo seasonal migrations where adults feed along the coast during summer into autumn after which they migrate to overwintering grounds in the deeper sub-basins (Aro, 1989). Adaptation of flounder in the Baltic Sea to the fluctuating salinity conditions has resulted in the differentiation of two spawning strategies: offshore spawning by flounder that produce large pelagic eggs that are only fertilized and retain neutral buoyancy at salinities above 10, and coastal spawning by flounder that produce smaller demersal eggs that can be fertilized at salinities as low as 6 (Nissling et al., 2002). Offshore spawning flounder occur mainly in the entrance area (SD 22–23) and the sub-basins of higher salinity (SD 24 to 26 and 28.2), but temporally also occur in the Åland archipelago (northern SD 29) and into the Gulf of Finland as a result of northward larval drift (SD 32) (Fig. 1). Coastal spawning flounder have the highest abundancies in the central, northern and eastern sub-basins (SD 25–30 and 32). No flounder of either of the two spawning strategies have been found to occur in the northern Gulf of Bothnia (SD) and easternmost Gulf of Finland (SD 32) (Bagge, 1981; Momigliano et al., 2019). Apart from the shared feeding and overwintering areas as adults in the central sub-basins (SD 25–28), individuals from the two spawning strategies share the same nursery areas as early juveniles, mainly shallow sandy bays <1 m depth (Florin et al., 2009; Martinsson and Nissling, 2011). The dispersal patterns of the hatched larvae of the two spawning types differ, with extensive dispersal for larvae of offshore spawners towards the nursery areas (Hinrichsen et al., 2018), and limited local dispersal for larvae of coastal spawners (Corell and Nissling, 2019). However, larvae of both spawning types use the same water layer (0–20 m depth) during the pelagic stage (56 ± 13 days from hatching to settling; see (Corell and Nissling, 2019).

Individuals of the two spawning types can potentially produce viable larvae together that do not differ in size from non-hybrids (Wallin, 2016), but no genetic evidence for natural hybridization has yet been found (Florin and Höglund, 2008; Momigliano et al., 2017). Recent studies indicate that the two spawning types can be considered a parapatric species pair (Momigliano et al., 2017, 2018). The coastal spawning type has been assigned to a new species, the Baltic flounder *P. solemdali* sp. nov., based on the distinct egg and sperm characteristics and strong reproductive isolation (Momigliano et al., 2018). Unfortunately, the two species cannot be distinguished based on any external morphological characteristics (Momigliano et al., 2018). The absence of a reliable morphological feature that can be used as a discriminant is especially complicated for species identification of larval and juvenile flounder, which can only be identified to species using genetic techniques. A feature or structure that is neither resorbed nor degenerates over time, and that can be used to reliably identify flounder to species in the Baltic Sea at multiple life phases, would be of great use to understand the demographics of the two co-occurring flounder species.

Otoliths, the calcified “ear stones” of teleost fishes, are good candidate structures for species identification. The acellular otoliths are metabolically inert and the incremental growth of otoliths enables analysis of the changes in chemical composition of an otolith at different life stages of the fish, from larva to adult (Campana and Thorrold, 2001; Pannella, 1971). The chemical composition of otoliths is dependent on the physical and chemical properties of the environment and the physiological state of the fish (Campana, 1999; Izzo et al., 2016; Loewen et al., 2016; Thomas et al., 2017). Various elements are incorporated differently into the otolith under the influence of both exogenous and endogenous factors (Campana, 1999; Hüssy et al., 2020; Izzo et al., 2018; Thomas and Swearer, 2019). The concentration of elements in otoliths can reflect the water chemistry of the external environment, but incorporation of these elements into the otolith can be influenced by ambient parameters such as temperature, salinity, pH and dissolved oxygen (Campana, 1999; Elsdon and Gillanders, 2003; Hüssy et al., 2020; Izzo et al., 2015; Thomas and Swearer, 2019). For example, temperature affects most physiological processes, including the biomineralization of otoliths, and changes in temperature may thus affect the precipitation of elements onto the mineral fraction of the otolith (Campana, 1999; Fablet et al., 2011; Hüssy et al., 2020; Sturrock et al., 2015). Furthermore, temperature influences the metabolic activity and, as a consequence, the influx of essential protein-bound elements into the endolymph fluid surrounding the otolith and subsequent incorporation of these elements into the organic matrix (Loewen et al., 2016; Thomas et al., 2017). Besides the exogenous factors that affect the otolith chemistry, variability in development, maturation or growth rates between individuals can be reflected in otolith chemistry signatures as a result of ontogenetic differences in protein synthesis or mineral precipitation rates (Chittaro et al., 2006; Hüssy and Mosegaard, 2004; Sturrock et al., 2014). In addition, a maternal influence on the chemical composition of the core has been found in several studies (DiMaria et al., 2010; Hegg et al., 2019; Loeppky et al., 2018; Thorrold et al., 2006; Volk et al., 2000), which suggests that maternal transfer of material and subsequent incorporation into each primordium (initial structure of the otolith) can occur in the embryo.

Chemical analyses have detected 50 elements incorporated into the otolith (Campana, 1999), but only a select few are included in this study. Strontium (Sr) and barium (Ba) are two elements that randomly substitute for calcium in the crystalline lattice of the otolith and are therefore considered as elements whose concentrations in the otolith reflect the ambient environmental concentrations (Bath et al., 2000; Doubleday et al., 2014; Thomas et al., 2017). Concentrations of Sr, which is released in the water column through weathering and dissolution of bedrock material, are generally higher and relatively constant in marine environments and lower in freshwater environments (Walther and Limburg, 2012). Ba concentrations often increase at salinities between 5 and 20 when Ba desorbs from organic particles of freshwater origin, after which it remains as free ions in the water column before it either precipitates as barite ($BaSO_4$) or is taken up by aquatic organisms (Martinez-Ruiz et al., 2019; Walther and Limburg, 2012). Magnesium (Mg) is also randomly trapped in the crystal lattice of the otolith, but incorporation has been found to be reflective of metabolic activity, not of ambient concentrations (Limburg et al., 2018; Miller, 2011; Woodcock et al., 2012). Other elements, such as copper (Cu) and zinc (Zn), play an important role in enzymatic processes that are involved in the biomineralization of otoliths (Thomas et al., 2019) and are incorporated into the organic matrix of the otolith (Izzo et al., 2016; Miller et al., 2006). However, exposure to elevated concentrations of these two elements and other heavy metals can result in increased element:Ca levels in otoliths, which has been found in fish from water bodies adjacent to former mining operations (Friedrich and Halden, 2010; Søndergaard et al., 2015) or industrial areas (Andronis et al., 2017; Ranaldi and Gagnon, 2010). The element manganese (Mn) is likely incorporated into the crystalline matrix of the otolith as a substitute of Ca (Thomas et al., 2017), but is also a co-factor of biomineralization enzymes (Thomas et al., 2019). Otolith Mn:Ca is therefore considered under influence by both exogenous and endogenous factors (Limburg and Casini, 2018). Mn is a redox-sensitive element that dissolves and releases into the water column under low-oxygen conditions (Burdige, 1993; Tebo et al., 2004). Prolonged exposure of fish to high ambient Mn concentrations is reflected in the otolith by increased Mn concentrations (Limburg et al., 2015). In addition, the otolith elemental composition can show seasonal cycles and ontogenetic trends, which can reflect patterns of somatic growth, body condition, and even reproductive investment (Sturrock et al., 2014, 2015).

Otolith chemistry methods have been used in multiple studies to delineate stock units or discriminate contingents (Tanner et al., 2016). The life history information retrieved from otolith chemistry analyses can confirm the evolutionary information provided by genetic analyses when the delineation of a population using otolith chemistry is resolved into similar units as when genetic markers are applied (e.g. Campana and Thorrold, 2001; McKeown et al., 2015; Milton and Chenery, 2001a; Woods et al., 2010). The inclusion of otolith chemistry can improve the spatial resolution of population and stock assessments through identification of components or contingents that were obscured due to intermixing at the adult stage or straying at the reproductive stage (Barnett-Johnson et al., 2010; Barton et al., 2018; Martin et al., 2015; Steer et al., 2009). Otolith chemistry has even been able to segregate subpopulations or breeding stocks that were considered as genetically homogenous or constant, such as for Atlantic cod in the Kattegat-Öresund area of the eastern North Sea (Svedäng et al., 2010) and for the coporo *Prochilodus mariae* from the Apure drainage of the Orinoco basin in Venezuela (Collins et al., 2013). Otolith microchemistry therefore has the potential to emphasize differences in ecology between the two flounder species in the Baltic Sea that cannot be captured by genetic identification analyses of these weakly separated species.

The reliable identification of each individual flounder from the Baltic Sea to the correct species would require otolith microchemical signatures that are distinct between the two species. Only a few studies have investigated and successfully demonstrated the applicability of otolith chemistry for species discrimination (Chang and Geffen, 2013; Hamer and Jenkins, 2007; Radigan et al., 2018). Intrinsic species-specific differences in physiology, elemental transport mechanisms or blood chemistry may cause taxonomic differences in otolith chemistry signatures, even when taxa reside in (nearly) identical habitats throughout their entire life history or are exposed to the same environmental conditions. Spatially consistent differences in the otolith chemistry have been identified between juveniles of three flatfish species, including the European flounder *P. flesus*, that co-occurred in the same estuarine habitats along the Portuguese coast (Reis-Santos et al., 2008). Otolith chemistry could therefore conceivably be used as a potential tag in the discrimination of the two flounder species in the Baltic Sea. However, the complex hydrological regime of the Baltic Sea leads to spatial differences in environmental conditions in the sub-basins of the central Baltic where the distribution of both species overlaps. Biomineralization of otoliths is under physiological control and is influenced by the physical and chemical properties of the environment, which means that otolith chemistry signatures of individuals from separate locations could reflect the spatial variability in environmental, ontogenetic, and growth conditions (Campana, 1999; Hüssy et al., 2020; Thomas and Swearer, 2019). The spatial variability may be large enough that potential species-specific differences are concealed through the environmental and physiological control on the chemical composition of otoliths. However, even if individual otolith chemistry signatures are influenced by local habitat conditions, the relative differences in otolith chemistry signatures between the two species may be maintained across different sub-basins. The validity of otolith chemistry as a reliable tool for species discrimination of flounder from the Baltic Sea is therefore dependent on whether or not differences in otolith chemistry signatures between the two species are constant across the entire shared distribution. Therefore, the objectives of this study are:

1) to determine if there are differences in otolith elemental signatures between the European flounder *P. flesus* and Baltic flounder *P. solemdali* in the central Baltic Sea;
2) to investigate if any potential differences in otolith chemistry signatures between the two flounder species are consistent across the region where the distributions of both species overlap within the Baltic Sea; and,
3) to assess potential geographical differences in otolith chemistry signatures.

We focused on the early life stages - embryos, larvae and early juveniles - that cannot be separated based on reproductive traits. We tested for the existence of species-specific otolith chemistry tags for both flounder species and the consistency of these tags between three survey areas that are located along a geographical gradient from SW to NE in the central Baltic (SD 25, 26 and 28). In addition, we investigated the relative influence of two sources of elemental incorporation, the maternal transfer of otolith material during the embryonic phase and the post-hatching environment of the larval and early juvenile phases, on the otolith chemistry signatures of age 0-group juvenile flounder. Finally, reclassification procedures were conducted to test the accuracy of the application of otolith chemistry signatures for species discrimination and area of origin discrimination of flounder in the Baltic Sea.

2. Materials & methods

2.1. Sample collection

Otoliths from age 0-group juvenile flounder were acquired from different surveys within the BONUS INSPIRE project (https://www.bonus-inspire.org/) that were conducted in the Baltic Sea in 2014 and 2015. Juveniles were sampled at nursery areas (shallow sandy beaches) along the Swedish and Latvian coast in 2014 (Fig. 2).

Sampling took place at two locations along the Swedish embayment of Hanö Bay, two locations along the coast of the Swedish island of Gotland, and two locations along the southern coast of the Latvian region of Courland. Age-0 juveniles were collected from early July to early September using beach seines with a mesh size of 4 mm. Individuals were stored immediately in 95% ethanol and sagittal otoliths were

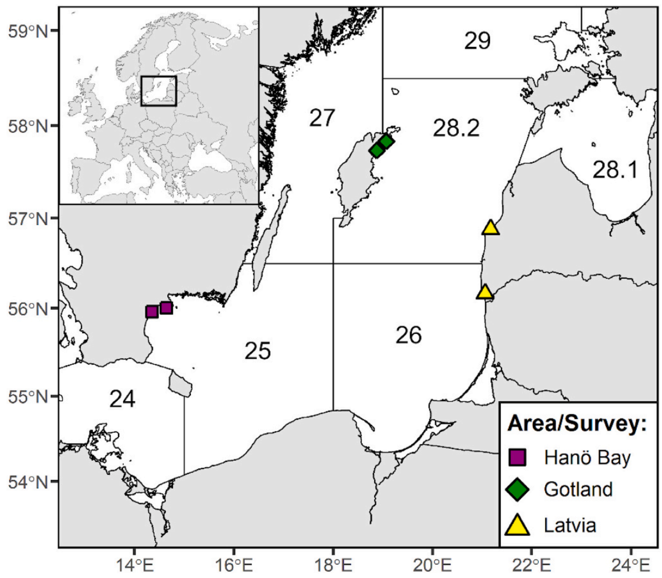

Fig. 2. Sampling sites of age-0 group flounder (*Platichthys* spp.) in the Baltic Sea. Sites from different surveys that were conducted in the same sampling area have identical symbol shapes.

extracted in the labs of Uppsala University for the Swedish specimens and the Institute of Food Safety, Animal Health and Environment (BIOR) for Latvian specimens. Tissue samples were taken during otolith extraction in the laboratory. Samples were sent to the Fish Genetics Laboratory (FiskGen) of the Institute of Freshwater Research of the Swedish University of Agricultural Sciences (SLU) for genetic species diagnostics.

2.2. Otolith preparation and chemical analyses

Sagittal otoliths were cleaned and then embedded in Struers EpoFix epoxy resin. Otoliths were polished down to the core in the sagittal plane to create thin sections. Prepared otolith sections were mounted on petrographic slides and cleaned with 95% ethanol before chemical analysis. A total of 278 otoliths were analysed for this study (Table 1).

Otolith chemistry analyses were performed using laser ablation inductively coupled mass spectrometry (LA-ICPMS) at the Analytical and Technical Services laboratory of SUNY-ESF. A New Wave (ESI) 193-nm laser system ablated otolith material along a prescribed transect from edge to edge through the core following the longest axis (Fig. S1). Laser parameters for analysis were set to a 35 μm spot size, 3 μm s^{-1} travel speed, 10 Hz wave frequency and 90% power. The ablated material was transported via a helium carrier gas into a PerkinElmer ELAN DRC-e quadrupole ICPMS to measure element concentrations.

Table 1
List of number of otoliths from age-0 juvenile flounder included in chemistry analyses (LA-ICPMS) and the number of otoliths used in statistical analyses based on chemistry and genetic data. Number of otoliths per species (*P. flesus* and *P. solemdali*) is given. Number of otoliths used for statistical analyses are given for core chemistry analyses (maternal influence) and for analyses outside the otolith core to the edge (post-hatching phase).

			Statistical analyses	
Area	Species	LA-ICPMS	Otolith core	Otolith post-hatching phase
Hanö Bay	*P. flesus*	36	26	29
	P. solemdali	42	28	31
Gotland	*P. flesus*	20	10	13
	P. solemdali	83	67	70
Latvia	*P. flesus*	41	29	27
	P. solemdali	56	40	40

The elements included in this study were: ^{24}Mg, ^{43}Ca, ^{55}Mn, ^{63}Cu, ^{64}Zn, ^{88}Sr, ^{127}I, ^{138}Ba, and ^{208}Pb. In short, Sr and Ba provide information about salinity, habitat use and migration of fish (Elsdon et al., 2008; Walther and Limburg, 2012). Mg incorporation can be used to identify seasonal growth patterns (Limburg et al., 2018). Mn and I are indicators of exposure to low oxygen conditions (Limburg et al., 2015; Lu et al., 2010). Incorporation of Cu, Zn and Pb is mainly regulated by endogenous processes (Hüssy et al., 2020; Thomas and Swearer, 2019), but exposure to elevated concentrations can also be reflected in otolith concentrations of these elements (Milton and Chenery, 2001b).

The MACS-3 carbonate standard developed by the U.S. Geological Survey was used for calibration and instrument drift corrections. Additionally, an in-house pressed pellet of powdered otoliths was used to calibrate for calcium (Limburg et al., 2011). Precision, expressed as the percent relative standard deviation (%RSD), was estimated using the MACS-3. The following average values of %RSD were calculated based on 95 MACS-3 runs: ^{24}Mg, 14.7%; ^{43}Ca, 10.8%; ^{55}Mn, 13.2%; ^{63}Cu, 15.0%; ^{64}Zn, 18.5%; ^{88}Sr, 14.0%; ^{127}I, 29.1%; ^{138}Ba, 15.5%; and ^{208}Pb, 15.9%. The non-homogenous composition of the MACS-3 could explain the deviations in element concentrations. Rerunning ablation transects on otoliths produced nearly identical results in terms of magnitude and pattern reproducibility of molar trace element to Ca ratios.

2.3. Genetic analyses

Sixteen microsatellite markers were used to genotype individuals (Florin et al., 2017). The software program STRUCTURE 2.3.4, which implements a Bayesian approach by applying a Markov Chain Monte Carlo algorithm, was used to assign individuals to genetic clusters (Pritchard et al., 2000). Two distinct clusters were identified that corresponded to the two species, which allowed individuals to be probabilistically assigned to a species (Florin et al., 2017). Only individuals that were assigned to a single species with a probability of at least 0.75 were included in the data analyses (81.3% of individuals).

2.4. Data analyses

Trace elemental concentrations (ppm) measured along transects were standardized by expressing the concentrations as elemental ratios to Ca. Ratios were base-10 log-transformed to improve statistical performance by approximating a normal distribution. Average values of trace element-to-calcium ratios across the portion of the otolith of interest were calculated and used for statistical analyses. Analyses were performed with R version 3.6.1.

2.4.1. Chemical profiling of species and sampling areas

Otolith chemical signatures were compared between the two species and/or among the three sampling areas using multivariate analyses of variance (MANOVA). Species and area differences were tested for significance using Pillai's trace test statistic. Principal component analyses (PCA; R packages factoextra and ggbiplot) were conducted to investigate the overlap between otolith elemental signatures of the two species and between elemental signatures of flounder (unassigned to species) from the three areas. The degree of overlap was calculated between 95% confidence interval ellipses centred on group means of the first two principal components (R package SIBER). Differences in the otolith elemental signatures were further investigated at a single-element level using univariate analyses of variance (ANOVA) with post-hoc comparisons of least square means for both species and area discrimination.

Elemental signatures of the age-0 otoliths were subdivided in a maternally influenced signature and a post-hatching signature. Maternal transfer of otolith material components to the embryos results in the incorporation of elements into the core zone of the otoliths of offspring. Signatures of Cu and Zn often show a peak in the core (MS and KL, unpublished observation) and were used to identify the location of the core. All peaks of these two elements declined to constant levels within the first 50 μm of the core. The transect portion of the otolith within 50 μm of the core was defined as the maternally influenced otolith zone. Elemental ratio values for the core zone were averaged across a region of 50 μm on each side of the core (100 μm total). The transect portion outside the core region to the edge of the otolith was defined as the post-hatching zone of the otolith. The longest transect of core to edge often had the highest resolution and was selected to calculate the average elemental ratio values for the post-hatching zone. Individuals with multivariate outliers in otolith chemical signatures were removed from statistical analyses when the Mahalanobis distance was >2 standard deviations away from the mean of the multivariate distribution (Rodrigues and Boente, 2011). Exclusion of multivariate outliers resulted in a final sample size of 200 otoliths for statistical analyses of otolith core chemistry signatures (maternal influence) and 210 otoliths for statistical analyses of otolith chemistry outside the core zone (post-hatching phase) (Table 1).

2.4.2. Reclassification based on chemistry signatures

The effectiveness of otolith chemistry signatures to discriminate between individuals of the two species and/or the three natal areas was tested through reclassification analyses. Random forest (RF) models were used to predict the membership of an individual to predefined classes (in this study the two species or three areas of origin) based on the otolith chemistry signature (Breiman, 2001). R packages randomForest and rfUtilities were used in the construction of the RF models.

The capacity of the RF model to correctly predict the species or area of origin of each test individual based on its otolith chemistry signature was evaluated. Two validation metrics were used to assess the predictive performance of an RF model: the macro F1 score and the Cohen's kappa coefficient (further details in Appendix). All combinations of otolith elemental variables were tested, resulting in $2^8 - 1 = 255$ combinations. The predictive performance of the RF for each variable combination was improved through 20-fold cross validation using different randomly divided training and test subsets for each RF. Macro F1 and Cohen's kappa scores were averaged over these 20 folds. Elemental variable combinations were ranked according to the average macro F1 score and the combination with the highest macro F1 score was selected as the best predictive model. The average Cohen's kappa score was used to further assess the reliability of top-ranked models to correctly reassign individuals to species or area of origin based on their otolith chemistry signature.

3. Results

3.1. Otolith chemistry of core zone (maternally influenced)

Otolith core chemistry multivariate signatures, reflecting maternal transfer to offspring, differed significantly between species ($F_{1,\ 200} = 4.13$, $p < 0.001$) and among areas ($F_{2,\ 200} = 10.32$, $p < 0.001$), but the interaction between species and area is not significant ($F_{2,\ 200} = 1.27$, $p = 0.213$). However, otolith core chemical signatures could not distinctly separate the age-0 juveniles by species: the 95% confidence interval of the first two principal components of *P. flesus* overlaps by 79% with that of *P. solemdali* (Fig. 3A). Individuals were also difficult to distinguish by their area of origin using otolith core chemistry: overlap between each pair of 95% confidence interval ellipses was at least 74% (Fig. 3B).

Scaling down to the level of species by area revealed that significant species differences are only found for the southern Latvian coastal waters (Fig. 4, Table S1). Otoliths from *P. solemdali* caught along the southern Latvian coast were distinct from those of *P. flesus* in log10-transformed ratios of Cu:Ca ($p < 0.001$), Mg:Ca ($p = 0.002$), Pb:Ca ($p = 0.040$), and Zn:Ca ($p = 0.027$). Latvian otolith cores also differed significantly in average log10 Mn:Ca ratios compared to Hanö Bay ($p < 0.001$) and Gotland ($p < 0.001$) for both species (Table S1). Back-transformation of the Mn:Ca ratio indicated that otolith cores from Latvian age-0 juveniles had higher Mn:Ca compared to those from age-0 juveniles from Hanö Bay and Gotland (Fig. 4).

Reclassification of individuals to species using otolith core chemistry signatures resulted in a maximum macro F1 score of 0.618 ± 0.015, but the Cohen's Kappa score of 0.209 ± 0.027 indicated that there is only slight agreement between the predictive species classification and the a priori species assignment (Table S2). The maximum macro F1 score for reclassification of individuals to area of origin was similar (0.620 ± 0.014) to that for species reclassification, but the predictive capacity based on Cohen's Kappa was higher for reclassification to area of origin (0.456 ± 0.020 = moderate agreement, Table S2). Subdivision of species by area of origin reduced the maximum macro F1 score to 0.378 ± 0.016 and agreement between class predictions and class observations did not improve (Cohen's Kappa of 0.336 ± 0.014 = fair agreement, Table S2).

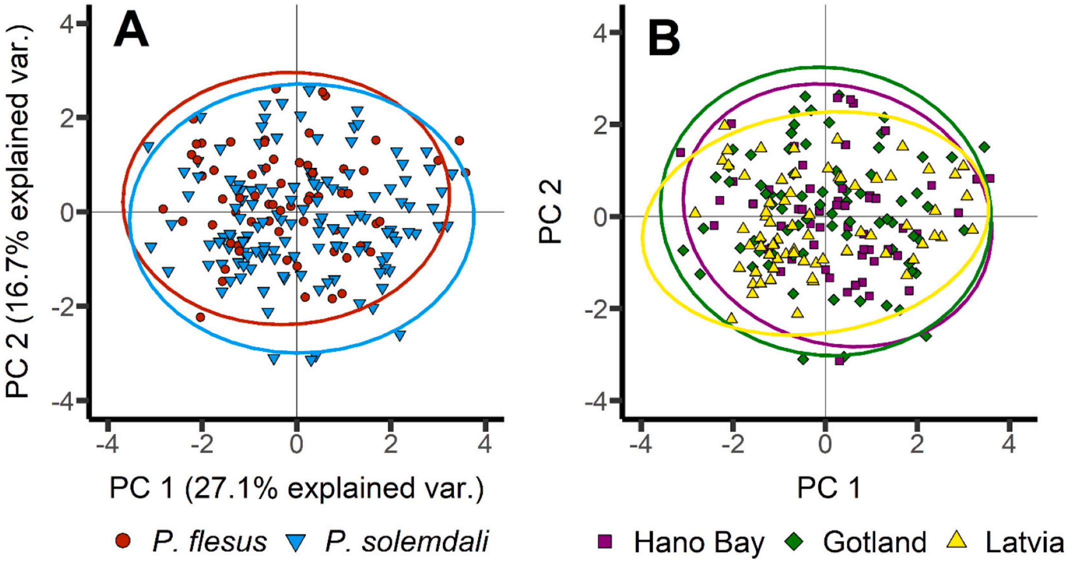

Fig. 3. Plots of the first two principal components of a PCA from otolith core chemistry data of age-0 group flounder. The percentage of variance explained by each principal component given in plot A also applies to plot B. Individuals are grouped by species (A) or by areas (B). Ellipses give the 95% confidence intervals of the principal component loading.

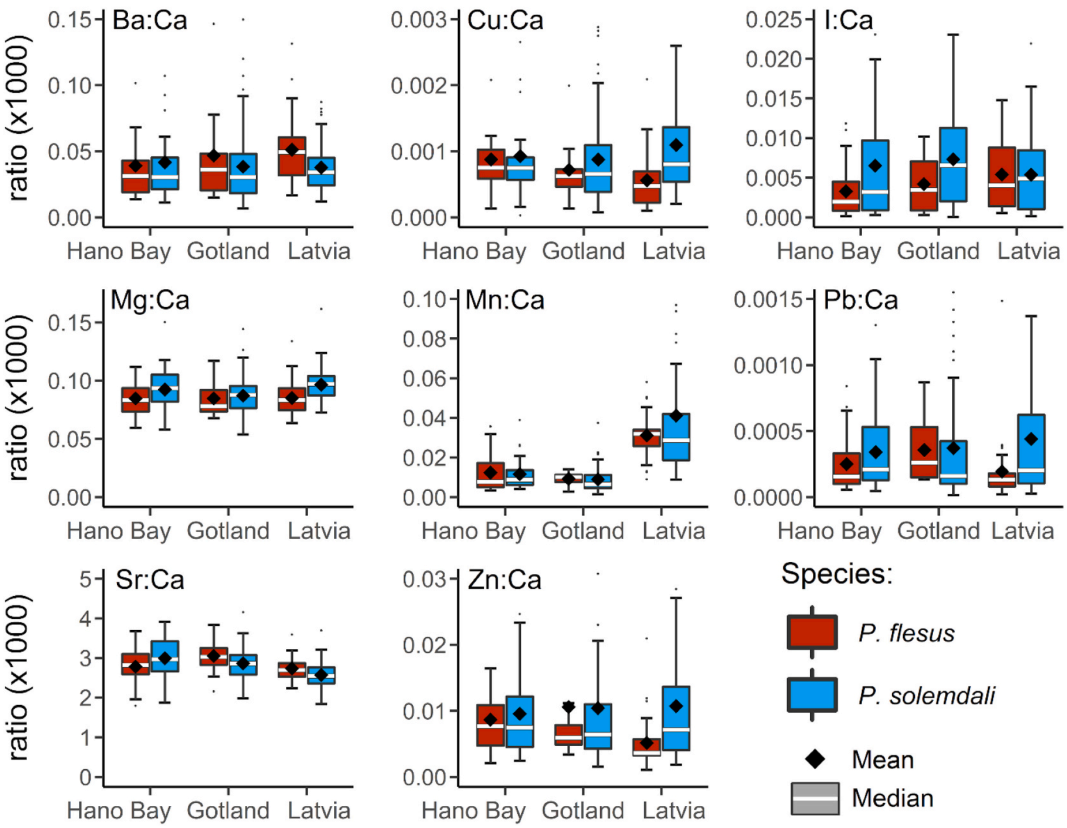

Fig. 4. Distribution of calculated averages of trace element-to-calcium ratios from the core zone of age-0 group flounder otoliths. Ratios are back-transformed to arithmetic scale. Species-specific values are displayed for each area of origin. Group-wise means and medians and the 25th and 75th percentiles of ratios are indicated.

3.2. Otolith chemistry of post-hatching phase

Chemistry analyses outside the otolith core to the edge, reflecting elemental incorporation during the post-hatching phase, indicated significant differences in multivariate elemental signatures between species ($F_{1,\ 210} = 8.04$, $p < 0.001$), between areas ($F_{2,\ 210} = 13.87$, $p < 0.001$), but not for the interaction between species and area ($F_{2,\ 210} = 1.43$, $p = 0.084$). The significant species difference found with the MANOVA was not reflected in the PCA analysis: there was a 67% overlap between the 95% confidence intervals of the two species (Fig. 5A). In contrast, separation of individuals based on their otolith chemical signature did improve when individuals were grouped by area (Fig. 5B).

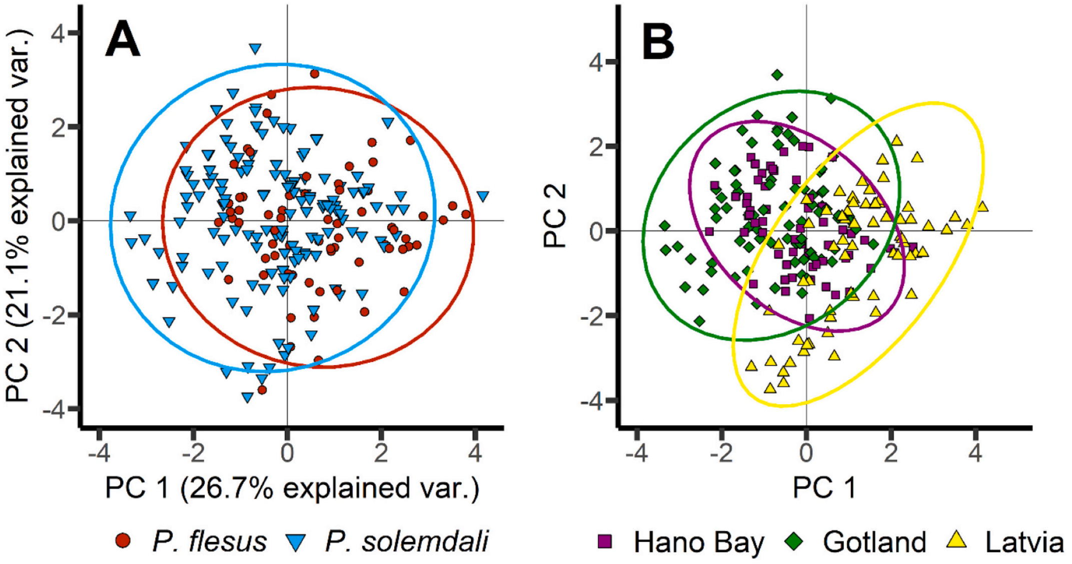

Fig. 5. Plots of the first two principal components of a PCA from otolith chemistry data of the zone reflecting the post-hatching phase from age-0 juvenile flounder. The percentage of variance explained by each principal component given in plot A also applies to plot B. Individuals are grouped by species (A) or by areas (B). Ellipses give the 95% confidence intervals of the principal component loadings.

However, categorization of individuals to area of origin mostly resulted in the separation of Latvian age-0 juveniles from those of Hanö Bay and Gotland: the 95% confidence intervals for Latvia and Hanö Bay and for Latvia and Gotland overlapped by 31% and 26% respectively. There was no clear partitioning between Hanö Bay and Gotland: the 95% confidence intervals for Hanö Bay and Gotland overlapped by 55%.

The distinction between Latvian age-0 juveniles and those from Hanö Bay and Gotland was reflected for several elements when univariate comparisons between areas were conducted (Fig. S2, Table S3). Significant differences in log10-transformed element ratios between Latvia and Hanö Bay and between Latvia and Gotland were found for Ba:Ca, I:Ca, Mg:Ca, Mn:Ca and Sr:Ca (all: $p < 0.04$). In addition, age-0 otoliths from Hanö Bay also differed significantly from those from Gotland in log10 Ba:Ca ($p = 0.006$), log10 Mn:Ca ($p < 0.001$) and log10 Sr:Ca ($p = 0.003$) (Table 2). Back-transformed Ba:Ca and Mn:Ca ratios showed the highest mean and median for Latvian individuals, with intermediate mean and median for individuals from Hanö Bay and the lowest mean and median for individuals from Gotland (Fig. S2). A downward trend in mean and median Sr:Ca was observed from Hanö Bay to the Latvian coast (Fig. S2).

Species differences in post-hatching chemistry were mostly identified for the Latvian coastal waters (Fig. 6, Table S4). Latvian otoliths of *P. solemdali* had significantly higher log10-transformed ratios of Cu:Ca ($p = 0.003$), Mg:Ca ($p = 0.002$), and Zn:Ca ($p = 0.046$) than those of *P. flesus*. In contrast, log10-transformed ratios were elevated for Ba:Ca ($p = 0.045$) and Sr:Ca ($p = 0.026$) in *P. flesus*. Hanö Bay individuals also had significant differences between *P. solemdali* and *P. flesus* in Mg:Ca ($p = 0.001$) and Mn:Ca ($p = 0.009$). However, there were no instances of significant differences between species for all three areas for any of the element-to-calcium ratios (Fig. 6, Table S4).

Reclassification of individuals to species based on their post-hatching chemistry signature indicated that the power to discriminate between the two species was low: the Cohen's Kappa score for the best combination of elements was 0.301 ± 0.020 (= slight agreement), even though the macro F1 score for that combination is 0.663 ± 0.010 (Table 2). Classification to species subdivided by area resulted in a higher Cohen's Kappa score (0.455 ± 0.018 = moderate agreement) for the best combination of elements, but the macro F1 score for that combination was lower at 0.468 ± 0.014. Predictive classification of individuals to area of origin led to the highest maximum F1 score of 0.804 ± 0.010. In addition, the best combination of elements had a Cohen's Kappa score 0.706 ± 0.015, which indicated good agreement between the predictive area classification and the a priori assignment to area of origin (Table 2). On average, 90.4% (RSE = 2.0%) of the Latvian age-0 juveniles were correctly reclassified as originating from Latvian coastal waters using the best combination of elements. The average percentage of Gotlandic age-0 juveniles that were correctly reclassified as originating from Gotland was determined at 81.1% (RSE = 2.2%), while 69.0% (RSE = 3.1%) of age-0 from Hanö Bay were correctly reclassified. Misclassification of individuals mostly occurred between individuals from Hanö Bay and Gotland: on average, 24.5% (RSE = 9.6%) of age-0 from Hanö Bay were reclassified as Gotlandic individuals, while 16.3% (RSE = 10.4%) of Gotlandic age-0 were on average reclassified as originating from Hanö Bay. Mn and Sr were included in all the top element combinations with the highest macro F1 scores for area of origin reclassification (Table 3). Ba was included in 84% of the top 25 element combinations for reclassification to area of origin.

Table 2

Element combinations ranked by macro F1 score through Random Forest analyses of age 0 otolith chemistry data of the zone reflecting the post-hatching phase. The top three combinations are displayed for predictive classification to species, to area of origin and to species subdivided by area of origin. Cohen's Kappa coefficients are given for each combination and cells are color-coded based on interpretation guidelines from Landis and Koch (1977) (red for <0.00–0.40 = poor to fair agreement; orange for 0.41–0.60 = moderate agreement; green for 0.61–1.00 = substantial to almost perfect agreement). Values given for both statistics are averages (± standard error) from 20 iterations.

	Rank	Combination of elements (in ratio versus Ca)	Macro F1 score	Cohen's Kappa
Species	1	Ba Cu Mg Pb	0.663 (± 0.010)	0.301 (± 0.020)
	2	Ba Cu Mg Mn Pb Sr	0.645 (± 0.012)	0.274 (± 0.024)
	3	Ba Cu Mg Mn Sr	0.638 (± 0.010)	0.262 (± 0.021)
Area of origin	1	Ba Cu Mn Pb Sr	0.804 (± 0.010)	0.706 (± 0.015)
	2	Ba Cu I Mn Sr	0.781 (± 0.011)	0.673 (± 0.016)
	3	Ba Cu Mn Sr	0.779 (± 0.012)	0.672 (± 0.017)
Species by Area of origin	1	Ba Cu Mg Mn Pb Sr	0.468 (± 0.014)	0.455 (± 0.018)
	2	Ba Cu Mg Mn Pb Sr Zn	0.466 (± 0.012)	0.449 (± 0.017)
	3	Ba Mg Mn Pb Sr Zn	0.455 (± 0.011)	0.445 (± 0.014)

4. Discussion

4.1. Species discrimination through otolith microchemistry

We found scant evidence that otolith chemistry could distinguish age-0 juvenile *Platichthys flesus* from *P. solemdali* across the entire study region in the central Baltic Sea. Multi-elemental signatures in the otoliths were not distinct enough to discriminate the two species. This was reinforced by the weak performance of the Random Forest algorithms for species reclassification. Species differences for single element:Ca ratios were detected within sampling areas, but these differences were not consistent across the entire study region. The southern Latvian coastal waters comprised the only sampling area for which ratios of multiple elements (Ba, Cu, Mg, Sr, and Zn) were different in their species-specific group-wise averages, with *P. flesus* having higher group-wise averages for Ba and Sr in the post-hatch zone and lower group-wise averages for Cu, Mg, and Zn across both otolith zones than *P. solemdali*. However, we found it difficult to identify species-specific differences at the sub-regional level, even in southern Latvia.

Otolith chemistry in the core region could reflect differences in abiotic conditions in spawning habitats (offshore vs. coastal) and physiological response to these conditions. The post-hatch otolith chemistry could be affected by the abiotic conditions experienced and physiological response to these conditions during the period of larval drift, subsequent settlement/metamorphosis and migration of age-0 juveniles to the nursery areas, and residence of these juveniles in these nursery areas until collection. Larval drift and vertical distribution patterns have to be taken into account in order to explain differences observed in the post-hatch chemistry. The majority of *P. flesus* larvae migrate vertically from deep areas below the permanent halocline at 50–80 m to the upper 30 m of the water column and drift for a period of 56 ± 13 days before settling (i.e. metamorphosis) and migrating into nursery areas (Hinrichsen et al., 2018). In contrast, larvae of *P. solemdali* will remain at a depth of 0–22 m after hatching until metamorphosis to ensure retention close to suitable nursery areas and prevent drift to open sea (Corell and Nissling, 2019). Arrival to nursery areas occurs in cohorts for both species from early July to early September (Martinsson and Nissling, 2011; Nissling and Wallin, 2020), which means that age-0 juveniles may originate from different areas. The more local larval dispersal of *P. solemdali* would imply that the individuals from this species included in this study would have likely spawned within the same area of the collection site. The extensive dispersal of *P. flesus* under the influence of surface currents makes it more difficult to determine the spawning location and drift pathway of the collected individuals. However, in a study on larval distribution patterns of *P. flesus* in the central Baltic Sea, it was found that most larvae that originated from the Arkona and Bornholm Basins (SD 24 and 25) would settle and migrate into nursery areas along the coasts of these sub-basins and in the entrance area (SD 22 and 23), whereas individuals that were spawned in

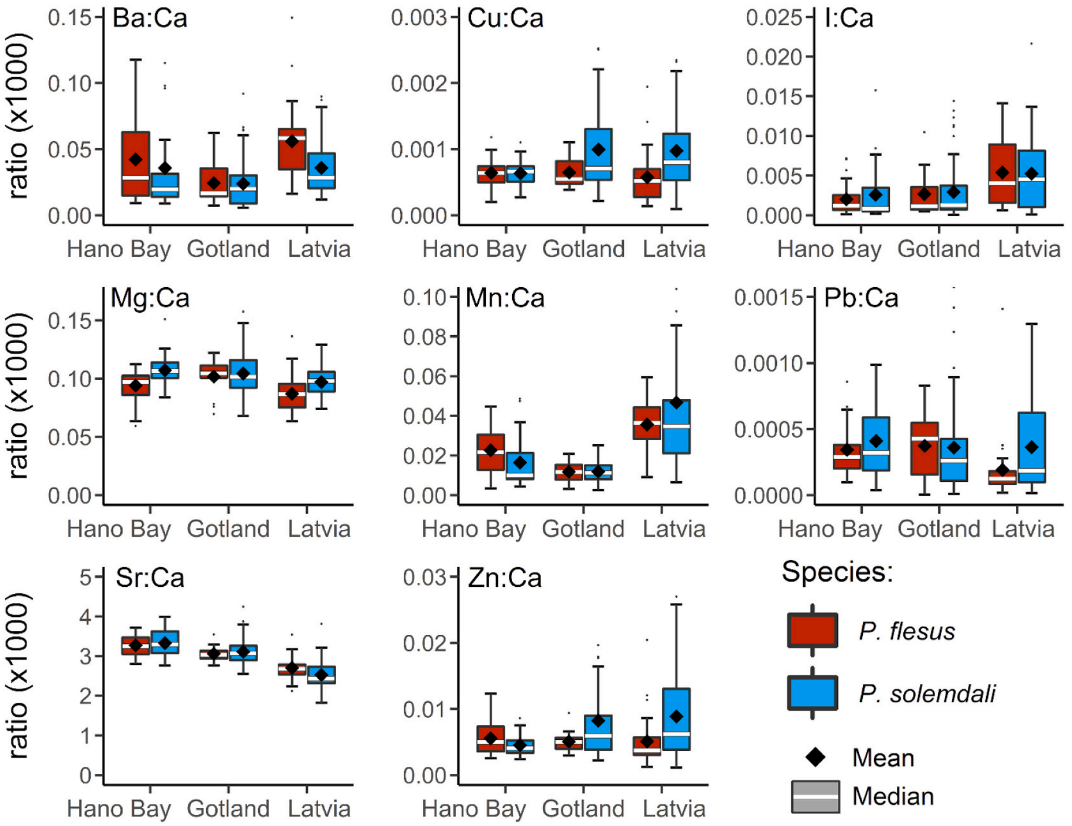

Fig. 6. Distributions of calculated averages of trace element-to-calcium ratios from zone reflecting the post-hatching phase for age-0 juvenile flounder otoliths. Ratios are back-transformed to arithmetic scale. Species-specific values are displayed for each area of origin. Group-wise means and medians and the 25th and 75th percentiles of ratios are indicated.

Table 3
List of percentage of occurrences of each element in the top 25 combinations for age-0 juvenile otolith chemistry data of the post-hatching phase. Combinations are ranked by macro F1 score through Random Forest analyses. Occurrences are displayed for predictive reclassification to species, to area of origin and to species divided by area of origin.

	Ba	Cu	I	Mg	Mn	Pb	Sr	Zn
Species	96%	84%	36%	80%	80%	76%	48%	36%
Area of origin	84%	64%	40%	32%	100%	52%	100%	40%
Species by Area of origin	76%	64%	36%	76%	96%	64%	100%	40%

the Gdansk and Gotland Basins (SD 26 and 28) would mainly utilize nursery areas along the east coast and islandic coasts of those basins (Hinrichsen et al., 2018). This would imply that the age-0 juveniles of *P. flesus* from Hanö Bay would have likely originated from the Bornholm Basin and that the age-0 juveniles of *P. flesus* from the sampling locations along the Latvian coast and on Gotland would have originated from both the Gdansk and the Gotland basin. However, the expected spatially segregated origins and drift pathways between *P. flesus* from Hanö Bay and *P. flesus* from Gotland and the Latvian coast are not reflected in the otolith chemistry signatures of age-0 juveniles.

The different elements that were included in this study are incorporated into the otolith under the influence of both exogenous and endogenous factors (Campana, 1999; Hüssy et al., 2020; Izzo et al., 2018; Thomas and Swearer, 2019). Strontium was selected in this study for its use as a conservative tracer within the Baltic Sea since strontium water concentrations reflect the salinity levels. Age-0 juveniles of the two flounder species had nearly identical ranges in average Sr:Ca, both for the maternally influenced otolith zone and the post-hatching otolith zone. Sr core chemistry can apparently not be used to distinguish the offshore spawning locations of *P. flesus* (spawning at higher salinities above the low-oxygen bottom waters) from the coastal spawning locations of *P. solemdali* with lower salinities (Nissling et al., 2017).

Barium was selected as the other element for which otolith chronologies reflect ambient concentrations and has been used in many fish ecology studies to determine movement and migration patterns of individuals across water bodies/masses (Elsdon and Gillanders, 2005a; Hamer et al., 2006; Secor et al., 2001; Smith and Kwak, 2014). Only *P. flesus* from Latvian coastal waters had higher Ba:Ca than *P. solemdali* and just for the post-hatching otolith zone. This leads to the open question of whether *P. flesus* larvae that settle along the Latvian coast encounter water masses with higher Ba levels during the drift period.

The other elements that were included in this study are incorporated into the otoliths under stronger influence of endogenous factors (Hüssy et al., 2020; Izzo et al., 2016; Thomas et al., 2017). The chronological patterns in otolith Mg:Ca at the juvenile and adult life stages correspond with seasonal patterns of otolith accretion, indicating that Mg is a tracer of somatic growth and metabolic activity (Grammer et al., 2017; Limburg et al., 2018; Sturrock et al., 2015). Otolith Mg:Ca has been observed to follow the visual otolith increment patterns in flounder from the Baltic Sea, with Mg:Ca minima located at translucent winter zones and maxima occur where daily growth increments are large enough to resolve (Limburg et al., 2018). These Mg:Ca patterns have mostly been identified in otoliths of adult flounder that have experienced multiple seasonal shifts in environmental conditions. The age-0 juveniles that were captured for this study only lived during the productive seasons of spring and summer. We did not find any significant differences in Mg:Ca between the two flounder species for their early life stages across the entire central Baltic Sea. However, differences in growth rate between the two species have been identified for adult flounder. (Nissling and

Dahlman, 2010) determined that *P. solemdali* had lower growth rates than *P. flesus*, which was related to higher reproductive investment by *P. solemdali*. Species-specific differences in Mg:Ca related to growth and indirectly to reproductive effort could potentially be found at later stages of flounder ontogeny.

The elements copper, zinc, and lead had the potential in this study to inform about species-specific differences in biomineralization and/or exposure to metal contaminated water masses or sediments. We did not find any differences in Cu:Ca, Zn:Ca and Pb:Ca between the early stages of the two flounder species across the entire Baltic study area. However, age-0 *P. solemdali* in Latvian coastal waters had a much greater variability in Cu:Ca, Zn:Ca and Pb:Ca than age-0 *P. flesus*, both for the maternally and post-hatching otolith zones. Laboratory experiments may elucidate the relative influence of exogenous factors (e.g., temperature) and endogenous factors (e.g., condition) on the incorporation of Cu, Zn and Pb in flounder otoliths, which may explain the individual variability in otolith concentrations that we have observed.

Otolith Mn:Ca was proposed as a tracer of low-oxygen conditions, also known as hypoxia (Altenritter et al., 2018; Limburg et al., 2015; Limburg and Casini, 2018; Mohan and Walther, 2016). We observed peaks in otolith Mn:Ca indicating that age-0 juvenile flounder encounter low-oxygen waters. Elevated manganese concentrations have been found in the core zone of the otolith in several species (Brophy et al., 2004; Melancon et al., 2008; Ruttenberg et al., 2005). The formation of the otolith core occurs during the embryonic phase prior to active feeding and it has therefore been suggested that the constituents of the core have to be present in the egg and are not obtained from the environment (Weigele et al., 2017). Natural enrichment of the core with Mn has been attributed to maternal investment and (Ruttenberg et al., 2005) determined that core Mn concentrations were higher in species that produce larger eggs. In the age-0 juvenile flounder otoliths used in this study, elevated otolith Mn:Ca levels were mostly located outside the core, but no species differences could be inferred. We did observe small peaks of Cu and Zn that are lined up with the location of the core in transects. However, the two flounder species could not be distinguished based on core Cu:Ca or Zn:Ca. Furthermore, no other evidence of species-specific differences in maternal transfer could be found based on age-0 juvenile core otolith chemistry in this study.

Phylogenetic signals in otolith chemistry signatures have been identified for species that occupy the same region (Chang and Geffen, 2013). Although spatial variability in otolith signatures within species can obscure taxonomic signals, otolith chemistry responses to spatial changes in water chemistry can be similar for different species (e.g. positive correlation between otolith Ba and ambient Ba) (Hamer and Jenkins, 2007; Swearer et al., 2003). Most studies found that differences in otolith chemistry signatures were strongest between species or species groups that were phylogenetically more distinct and/or had different ecologies (Geffen et al., 1998; Jones and Checkley, 2017; Leakey et al., 2009; Swearer et al., 2003). However, two perciform species were easily discriminated from three flatfish species based on otolith chemistry signatures, but there were also interspecific differences in otolith chemical composition among the flatfish species within estuaries, even between two sister species of sole *Solea* spp. (Reis-Santos et al., 2008). In addition, a recent study on hybridization between plaice *Pleuronectes platessa* and flounder *Platichthys flesus* in the Inner Danish Waters found that over 70% of the juveniles were able to be correctly identified to species based on their otolith chemistry signatures (Brown et al., 2019). These two studies suggested that differences in micro-habitat use and physiological processes that regulate element incorporation could explain the differences in otolith chemical signatures between closely related species. However, none of the flatfish species in other studies are as closely related as the two flounder species in the Baltic Sea, which has been verified by weak genetic differentiation between the species (F_{ST} below 0.06) (Florin and Höglund, 2008; Momigliano et al., 2017). Genetic separation between the two species only started approximately 2400 generations ago and selection has been strongly associated with salinity (Momigliano et al., 2017, 2018). Adaptation of flounder in the Baltic Sea to the reduced salinity levels seems to have mostly influenced the reproductive and egg stages: differences between the two species have been found in spawning behaviour (Florin, 2005; Solemdal, 1970, 1971, 1973), fecundity and its regulation (Nissling et al., 2015; Nissling and Dahlman, 2010), sperm production (Nissling and Larsson, 2018) and propagule characteristics and activity (Nissling et al., 2002, 2017). In addition, the plasticity in movement behaviour across the estuarine gradient of metamorphosed juvenile flounder may have further obscured any species-specific signatures in otolith chemistry of the post-hatch phase. Ecological selection does not appear to have strongly affected life histories of early life stages of either flounder species in the Baltic Sea.

Experimental investigation of differences in otolith chemistry signatures between the two flounder species could be conducted using recirculating water tanks. In these experiments, various groups of flounder of both species would be exposed to different treatments of environmental parameters (e.g. salinity, temperature and oxygen level) and ambient concentrations of the elements included in this study. Even if these experiments would yield results that indicate species-specific differences in elemental incorporation into the otoliths, individuals in the field may not encounter the conditions that generate these otolith chemistry differences. Movement of fish across heterogeneous environments that also fluctuate in physical and chemical conditions over time is difficult to capture in experimental studies (Izzo et al., 2018). The complex hydrological conditions in combination with the strong variability in vertical and horizontal thermohaline structure of the Baltic Sea are difficult to replicate in laboratory and mesocosm experiments. In addition, exposure to certain elements may be under- or overestimated if important processes are not replicated in experiments. For example, manganese concentrations in water are dependent on redox conditions in the sediment and the absence or an insufficient representation of sediment exchange processes in experiments will thereby fail to replicate manganese exposure and subsequent otolith incorporation as experienced under field conditions, especially when investigating the effect of low oxygen conditions on manganese uptake (Mohan et al., 2014). The development of an effective tool for species discrimination of flounder in the Baltic Sea should therefore primarily focus on field-capture individuals.

The chemical composition isn't the only otolith characteristic that has been used in studies of species identification and population structure analyses: the otolith shape has also been applied as a marker to discriminate species and delineate populations and stocks (Afanasyev et al., 2017; Campana and Casselman, 1993; Midway et al., 2014; Stransky and MacLellan, 2005). Several studies where both otolith chemistry and otolith shape were used to determine assignment success of fish to stock or area of origin have indicated that the classification success is higher with otolith chemistry than with otolith shape (Avigliano et al., 2017; Longmore et al., 2010; Rodrigues Maciel et al., 2021). However, re-assignment of Atlantic spadefish *Chaetodipterus faber* to their area of origin in coastal south-eastern Brazil was more successful through application of a complementary approach than when only otolith chemistry or otolith shape analyses were applied (Soeth et al., 2019). A similar approach could be applied for species identification of flounder in the Baltic Sea, although no visible differences in otolith morphology between the two species were observed by the authors. Since both environmental and genetic factors can affect otolith shape (e.g. Vignon and Morat, 2010), any species-specific attributes of otolith shape that could be identified in prospective analyses need to stand out enough to overcome intraspecific variability in otolith shape for otolith morphology to be considered as an effective discrimination tool for Baltic Sea flounder species.

4.2. Natal origin discrimination through otolith microchemistry

Switching the focus to area of origin discrimination at a larger regional scale (i.e., 100s of kilometers) reveals that Latvian age-0 juvenile flounder can be distinguished from age-0 juveniles from Hanö Bay and Gotland based on their otolith chemistry. The relatively clear distinction in otolith chemistry signatures between Latvian age-0 juveniles and those from the other two sampling areas is mostly restricted to the chemistry of the post-hatching otolith zone. The degree of overlap in post-hatching multi-elemental signatures between Latvian age-0 juveniles and those from Gotland and Hanö Bay was <35%. Latvian otoliths had higher group-wise averages of Ba, I, and Mn and lower group-wise averages of Mg and Sr compared to otoliths from Hanö Bay and Gotland. Reclassification of age-0 juveniles to their area of origin was correct for almost all the individuals from Latvian coastal waters, with Mn and Sr identified as the most important elements for determining the area of origin of age-0 juveniles.

Strontium water concentrations reflect the salinity levels along the salinity gradient, with highest Sr concentrations in the southwestern entrance area and lowest Sr concentrations in the north and northeast of the Baltic Sea (Andersson et al., 1992). Several experimental studies have demonstrated that otolith Sr concentrations are strongly positively correlated to water Sr concentrations (Bath et al., 2000; Elsdon and Gillanders, 2002, 2005b; Miller, 2011). The otolith Sr:Ca of age-0 juveniles decreased from Hanö Bay to the Latvian coast, a trend that was stronger for the post-hatching zone than for the maternally influenced zone of the otolith. The negative trend in otolith Sr:Ca follows the SW-NE salinity gradient in the central Baltic Sea. Contrary to Sr, the age-0 juveniles could not be discriminated to area based on otolith Ba:Ca ratios.

The restricted bottom inflow of oxygen-rich water into the Baltic Sea causes the deeper basins to be depleted in oxygen (Meier et al., 2019; Omstedt et al., 2014), but hypoxia has also been increasing in the coastal areas throughout the Baltic Sea (Conley et al., 2011). Differences in otolith Mn:Ca have not been found between the two flounder species for the early life stages, but Latvian age-0 do have higher otolith Mn:Ca than age-0 from Hanö Bay or Gotland. Oxygen levels measured during the summer of 2014 at the Latvian coast were lower (average of 5.4 ml L^{-1} (LHEI, 2018)) than those measured along the coast of the survey areas of Hanö Bay and Gotland (average of 6.3 and 6.4 ml L^{-1} respectively (obtained from SMHI database “vattenweb” at https://vattenwebb.smhi.se/modelarea/)). However, the oxygen levels measured at the Latvian coast are well above the hypoxia threshold value of 1.4 ml L^{-1} (= 2.0 mg L^{-1}) and it remains inconclusive if the relatively small difference in ambient oxygen concentrations between Latvian coastal waters and the survey areas of Hanö Bay and Gotland can explain the more than two-fold differences in otolith Mn:Ca. The other redox-sensitive element that has been suggested as a candidate proxy of hypoxia, iodine, has been found to decrease in concentration in marine carbonates and otoliths during periods of low-oxygen conditions (Limburg et al., 2015; Lu et al., 2010). High Mn:Ca should therefore be contrasted by low I:Ca in otoliths, but average I:Ca in otoliths from Latvian age-0 is also much higher than in otoliths from Hanö Bay and Gotland age-0. The sources of Mn and I in otoliths of Latvian age-0 juveniles need to be investigated in order to elucidate the elevated concentrations of these elements in the Latvian otoliths.

4.3. Potential for flounder otoliths to record Baltic Sea climate change

Otoliths of flounders are useful chemical recorders of environmental conditions. Chemical signatures of the otolith zone during the post-hatching phase reflect the salinity conditions along the lateral gradient in the Baltic Sea and can confirm the exposure of early life stages to low oxygen concentrations. Climate change is expected to increase temperatures and precipitation in the Baltic Sea and reduce the inflow of oxygen-rich water from the North Sea (Andersson et al., 2015). Salinity in the central Baltic Sea has been projected to decrease by 1.5–2 units by the end of the 21st century due to increased riverine discharge and reduced inflow of saline water (Meier, 2015). The hypoxic area that is located primarily in the Gotland Basin (SD 26 to 28) of the central Baltic Sea is expected to expand up to 80,000 km^2 by the end of the 21st century with the present level of nutrient inputs (Meier et al., 2011). Deteriorating salinity and oxygen conditions may affect the spawning success of both *P. flesus* and *P. solemdali* (Nissling et al., 2002, 2017; Nissling and Wallin, 2020; Orio et al., 2017; Ustups et al., 2013). The increased occurrence and duration of low-oxygen conditions in the nursery areas could negatively impact growth, development and survival of post-settled larvae and age-0 juveniles. Otoliths of larval and age-0 juvenile flounder could potentially be used as indicators to track changes in salinity and oxygen conditions experienced by these early life stages and thus a tool in studying population dynamics of these parapatric species. However, more knowledge is needed about incorporation of trace elements into otoliths and habitat utilization by the two flounder species in the complex Baltic Sea system. Once this knowledge is acquired, the environmental histories derived from larval and early juvenile otolith chemistry could be used to improve models that estimate recruitment levels and population dynamics of both flounder species in the Baltic Sea. In light of the projected climate change in the Baltic Sea region, otolith chemistry has the potential to become a tool to identify essential nursery habitats for management and protection/conservation.

4.4. Conclusions

We show that otolith chemistry can be applied with higher success to classify age-0 juvenile flounder from the central Baltic Sea to their natal origin than to determine the species identity of individuals. We were not able to identify any differences between the two species in otolith chemistry signatures of early life stages that would suggest species-specific differences in micro-habitat use or in physiological histories. Our results indicate that otolith chemistry in age-0 juvenile flounder from the Baltic Sea reflects spatial variability in environmental conditions that outweighs any potential chemical signals from species-specific habitat use patterns and/or physiological controls.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seares.2022.102233.

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