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Connectivity in the early life history of sandeel inferred from otolith microchemistry

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ABSTRACT

Connectivity is a central issue in the development, sustainability and effectiveness of networks of Marine Protected Areas (MPAs). In populations with site attached adults, connectivity is limited to dispersal in the pelagic larval stage. While biophysical models have been widely used to infer early dispersal, empirical evidence through sources such as otolith microchemistry can provide a means of evaluating model predictions. In the present study, connectivity in the lesser sandeel, *Ammodytes marinus*, was investigated using LA-ICP-MS otolith microchemistry. Otoliths from juveniles (age 0) were examined from four Scottish spawning areas predicted to differ in terms of larval retention rates and connectivity based on past biophysical models. There were significant spatial differences in otolith post-settled juvenile chemistry among locations at a scale of 100–400 km. Differences in near core chemistry pointed to three chemically distinct natal sources, as identified by a cluster analysis, contributing to settlement locations.

1. Introduction

Connectivity, the exchange of larvae, juveniles or adults across a species' range (Palumbi, 2003) is important to population dynamics, as it mitigates against local mortality events affecting population resilience and persistence (Hastings and Botsford, 2006; Prince and Hilborn, 1998). The extent of connectivity is a central issue in the development, sustainability and effectiveness of networks of Marine Protected Areas (MPAs) (Almany et al., 2009; Christie et al., 2010; Sale and Kritzer, 2008; Shanks et al., 2003) as, if a population within a network is to be viable, it needs to be self-persistent, either supplied by self-recruited or locally retained individuals (Burgess et al., 2014). Simulations of MPA network design indicate that where there is 'source-sink' dynamics (areas of net export and import respectively; Pulliam, 1988) it is arguably more beneficial to protect propagule sources (Roberts, 1997), which in turn should have a positive impact on the wider population and fishery yield (Gaines et al., 2010). MPAs are expected to benefit fish species that show a high level of site fidelity, in particular those that are highly substrate-associated, as protection will reduce fishing mortality (Polunin, 2002). However, finding appropriate ways to assess population connectivity remains a challenge, particularly in such site-attached species where dispersal is mostly dependent on an elusive planktonic early life stage, but needs to be addressed prior to implementing spatial management measures such as MPAs (Sale et al., 2005).

Otolith microchemistry provides a powerful tool for examining life stage dispersal and connectivity in regions where significant spatial differences can be detected (Campana et al., 2000; Gillanders and Kingsford, 1996; Thorrold et al., 2001; Vasconcelos et al., 2007). Trace metals from the local environment are permanently incorporated into the crystalline matrix of the otolith as they accrete continuously, are metabolically inert and increments are unlikely to be subject to resorption (Powles et al., 2006; Wright, 1990). Although otolith microchemistry can reflect a combination of local environmental chemistry and individual physiology, the resulting elemental composition can create a unique chronological 'signature' that can be used as a natural tag (Campana and Thorrold, 2001; Elsdon et al., 2008) to distinguish location and infer ontogenic change. Otolith microchemistry has previously been used to discriminate between capture location and detect the habitat shift from pelagic to bentho-pelagic behaviour in the sandeel *Ammodytes tobianus* (Laugier et al., 2015). Microchemistry has also been used to infer differences in natal origin from analyses of the chemical composition close to the otolith core i.e. the portion formed during embryogenesis (Di Franco et al., 2012).

Past studies of natal microchemistry have followed two types of statistical approach. The first is a supervised learning approach which relies on establishing a baseline sample of pre-dispersal life stages of known origin, ideally sampled in all spawning or nursery areas, in order to assign post dispersal individuals of unknown origin to sources characterised in the baseline sample based on their elemental

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signatures (e.g. Gillanders, 2002; Wright et al., 2010). The second type follows an unsupervised learning method, where clustering of the near core region of the otolith provides information on the number of contributing larval sources and their distinct elemental signatures (e.g. Di Franco et al., 2012). While the first method has the potential to assign settled fish to known spawning area, conducting a fully representative large-scale sampling of marine larvae from all potential source areas to compose the baseline is expensive and likely to be incomplete, therefore the utility of this approach, though sound, is limited (Campana et al., 2000; Munch and Clarke, 2008; White et al., 2008). Clustering does not allow assignment of fish to spatially defined larval sources, however, identifying the contribution of larval sources to distinct settlement locations provides useful information on the spatial scale over which larval dispersal takes place (Di Franco et al., 2012). Therefore, the second approach was adopted in this study as it can find the most homogenous groups within the individuals of unknown origin, identifying contributing sources by assigning them to distinct clusters.

The present study uses otolith microchemistry to examine connectivity in the lesser sandeel, *Ammodytes marinus* (Raitt) a species where dispersal is limited to early life history stages (Wright, 1996). Sandeels are small, elongate planktivorous fish which form an important prey source for numerous fish, seabirds and marine mammals (Daan, 1989; Santos and Pierce, 2003; Wanless et al., 1998) and also support the largest industrial fishery in the North Sea. Due to their importance to the food web they are one of the key species considered in the Scottish MPA network (The Scottish Government, 2015). *A. marinus* spawn eggs into sand between December and January (Gauld and Hutcheon, 1990; Winslade, 1971) and the larvae hatch between February and May (Langham, 1971; Macer, 1966; Wright and Bailey, 1996). The duration of this pelagic stage lasts between 32 and 90 days (Jensen, 2001; Wright and Bailey, 1996; Wright, 1993) and later stages begin schooling before settlement in late May to June (Wright and Bailey, 1996; Wright et al., 2000). As they need to bury in sandy areas with a low silt content (Holland et al., 2005; Wright et al., 2000), the distribution of settled juvenile *A. marinus* is patchy (Jensen et al., 2011; Macer, 1965). Following settlement, sandeels are rarely found further than 15 km away from known habitat (Engelhard et al., 2008; van der Kooij et al., 2008; Wright, 1996) and the maximum distance travelled by tagged individuals displaced from grounds was only 64 km over 1–3 years (Gauld, 1990).

Biophysical models that couple particle tracking and sea circulation models provide another approach to infer early dispersal in marine organisms with a planktonic phase (Cowen et al., 2006; Gallego et al., 2007; Miller, 2007; Nolasco et al., 2013). Several biophysical models of sandeel larval transport have been produced, differing in horizontal and vertical resolution of the underlying hydrodynamic models and the accuracy of biological parameters for particle tracking (Berntsen et al., 1994; Christensen et al., 2008; Gallego et al., 2004; Proctor et al., 1998). While such models lack the finer resolution and more realistic larval behaviour traits present in newer generation models (Petrik et al., 2015), the 2D, 35 km resolution sea circulation model used by Proctor et al. (1998) was based on reported hatch locations, times and pelagic larval duration and even this simple model appeared to explain the distribution of juvenile aggregations just prior to settlement. Moreover, results from a finer scale 3D bio-physical model that used North Sea sandeel fishing grounds to infer hatch locations (Christensen et al., 2008) produced comparable results, and both studies suggested an average larval transport among aggregations of <100 km apart, with similar levels of regional retention.

In this study we inferred natal sources and area-specific patterns of dispersal in settled juvenile sandeels using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) to quantify otolith trace elemental composition. Four areas around Scotland were selected as biophysical models suggest that they represent locations which exhibit different larval dynamics, namely acting primarily as sources (the North Minch and Orkney; Proctor et al., 1998), a sink (Shetland; see Wright, 1996) or as retentive coastal zone (the Firth of Forth; Proctor et al., 1998; Christensen et al., 2008). Specifically we investigated: (1) the scale of geographical variation among capture locations from an analysis of the elemental signature at the settled region of the juvenile otolith, (2) the number of possible chemically distinct natal sources settling to grounds in Scottish waters by cluster analysis of near-core chemistry and, (3) the degree of overlap between larval and settled juvenile populations by characterisation of the cluster elemental composition. The resolved scale of connectivity was discussed in context of spatial management measures.

2. Materials and methods

2.1. Juvenile sample collection

Juvenile sandeel (*Ammodytes marinus*) <10 cm in length from the 2014 year class were collected from areas of known larval abundance around the coast of Scotland (the North Minch, $N = 23$; East of Orkney, $N = 27$; Shetland, $N = 30$; Fig. 1) between 15 July and 14 August by dredge survey. Sampling platforms included Marine Scotland Science (MSS) research and charter surveys, conducted by the *MRV Alba na Mara* and *FV Sunbeam FR487* respectively. Samples were supplemented by a further collection of fish from the Firth of Forth (Bell Rock, $N = 28$) in early December during MSS's winter sandeel dredge survey (Fig. 1). Sandeel were measured at sea to the nearest 0.5 cm length class before being frozen for analysis back at the laboratory.

2.2. Study area

Sample locations are sited within or close to areas currently subject to spatial management measures. In July 2014, the Scottish Government designated a network of 30 Nature Conservation Marine Protected Areas (ncMPAs), of which 3 were for sandeels: southern Shetland, north-west Orkney and Turbot Bank off the east coast (The Scottish Government, 2015; Fig. 1). The Firth of Forth site is currently protected by the north east UK sandeel fishery closure. Potential connectivity routes between the areas are determined by both ocean circulation and local hydrographic conditions. Circulation in our study area is predominantly unidirectional, where Atlantic waters flow from the west coast, mixing with more coastal waters before entering the North Sea to the east of Shetland and through the Orkney-Shetland region via the Fair Isle current (Fig. 1; also see Turrell et al., 1990, 1996), where they continue eastwards into the central north sea as the Dooley current (Dooley, 1974). This Atlantic inflow is governed by seasonal variation in westerly wind regimes (Furnes, 1980; Winther and Johannessen, 2006) with maximum inflow occurring in the winter months. The ocean circulation to the west of Orkney is known to connect to the narrow shelf area to the west of the Shetland Islands (Hill et al., 2008). The Scottish coastal current (SCC) carries water northward from the Minches, flowing along the northern reaches of Scotland and into the North Sea (Simpson and Hill, 1986).

2.3. Otolith preparation

All equipment was washed in 10% nitric acid solution prior to use. Sandeels were re-measured for total length (±0.1 cm), total weight (±0.01 g) and both sagittal otoliths removed under laminar flow conditions with fine-tipped ceramic forceps. Otoliths were decontaminated by sonication in Elga ultra-pure (>18 MΩ·cm) water and preserved dry in plastic trays. After confirming 0-group status by macrostructural analysis of age, one sagitta from each pair was mounted on the sagittal plane with a droplet of Araldite CY212 resin (Agar Scientific, UK) onto a coverslip attached to a glass slide, as detailed in Donohoe and Zimmerman (2010). Thin sections were prepared by polishing the otoliths to just above the primordium, using a lapping machine fitted with 0.5–9.0 μm Ultraprep™ diamond lapping film discs (Buehler, UK). After

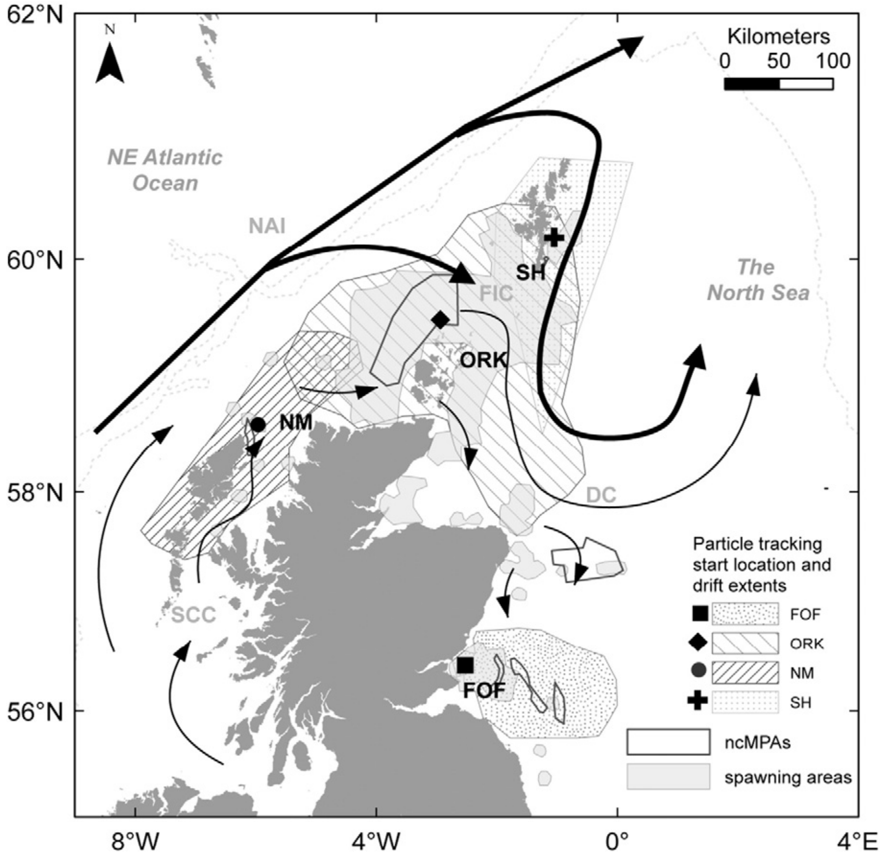

Fig. 1. Map of Scotland showing the four study locations (SH: Shetland; ORK: Orkney; NM: North Minch; FOF: Firth of Forth) and ncMPAs. Arrows broadly indicate prevailing surface ocean circulation, as adapted from Turrell et al. (1996), where the thick lines represent North Atlantic Inflow (NAI) and the thinner lines represent the more coastal currents such as the SCC (Scottish coastal current), FIC (Fair Isle current) and DC (Dooley current). Stippled/hatched areas represent the extents of sandeel larval drift up to 70 days post-hatch, from start locations within each study area (geographic mid-points of all start locations; black symbols), as predicted by Proctor et al. (1998).

polishing, the otolith sections were cut free from their slides, further decontaminated by sonication in Elga water, triple-rinsed and then left to dry under laminar flow. Polished sections were randomly mounted, to prevent sample batch bias, onto six glass slides in batches of 20 with a very thin layer of Glass Bond (Loctite™, UK) under the coverslips.

2.4. Otolith elemental analysis

Trace element composition was successfully conducted on 108 prepared sagittal sandeel otoliths from four areas by LA-ICP-MS in-house at MSS. Otoliths were analysed for the presence of 10 elements (^{7}Li, ^{24}Mg, ^{55}Mn, ^{65}Cu, ^{44}Ca, ^{88}Sr, ^{66}Zn, ^{85}Rb, ^{138}Ba, ^{208}Pb) using an Agilent 7700× inductively coupled plasma - mass spectrometer (Agilent Technologies Ltd., UK) equipped with a NewWave Research UP-213 laser ablation instrument, using helium gas as the carrier. External calibration was performed with a glass standard reference material (NIST 612; National Institute of Standards and Technology), with concentrations of each element being determined relative to this standard. A microanalytical carbonate standard (MACS 3; United States Geological Survey) was used to monitor the efficiency of analyte recovery.

All elements were expressed as ratios relative to ^{44}Ca, compensating for any variation in ablation yield between samples and standards, and are hereafter referred to as Li, Mg, Mn, Cu, Sr, Zn, Rb, Ba and Pb. Pre-ablation runs were undertaken on both standards and each otolith to remove any extraneous impurities. For each otolith, between 3 and 12 pits of 55 μm diameter were ablated in an even sequence along the maximum radius from the otolith core (hatch) to the outer edge (death). After every 10 otolith surface ablations, a helium blank, NIST and MACS ablation were also taken for calibration and instrument drift correction. Blank subtracted count data were gathered for each sample ablation in Masshunter software and converted to element concentrations ($\mu g \cdot g^{-1}$) in the otoliths by manual calculations using the internal standardisation equation described by Longerich et al. (1996). Elemental ratios with measures below background levels were considered as 0 (percentages of 0: 4.8 (Li), 0 (Mg), 0 (Mn), 20.4 (Cu), 0 (Zn), 7.8 (Rb), 0 (Sr), 0 (Ba) and 18.4 (Pb)). The mean percent relative standard deviations (% RSD) for NIST 612 standard glass during analyses were: 7.4 (Li), 5.6 (Mg), 4.5 (Mn), 10 (Cu), 9 (Zn), 6.7 (Rb), 6.1 (Sr), 9.1 (Ba) and 13.1 (Pb).

2.5. Otolith microstructure and microchemistry data

Microstructural analysis of the post-ablated otoliths was conducted in order to associate the microchemical results with life stage. Greyscale images of sagittae were captured under transmitted light using a Zeiss Axioskop 2 Plus light microscope, linked to a camera and image analysis software (Image Pro Insight; Media Cybernetics, UK) at magnifications ×20–×100. As the appearance of two accessory primordia (anterior and rostral; Wright, 1993) corresponds with metamorphosis, ablation pits were distinguished into a larval and settled juvenile sample per individual (Fig. 2 main), and age at metamorphosis estimated from otolith increment counts from a subsample of aged individuals ($N = 31$). Measurements (±0.01 μm) were taken from the primordium to the mid-portion of each ablated pit, along the otolith's maximum radius. Areas of otolith approximately a month both post-hatch and following

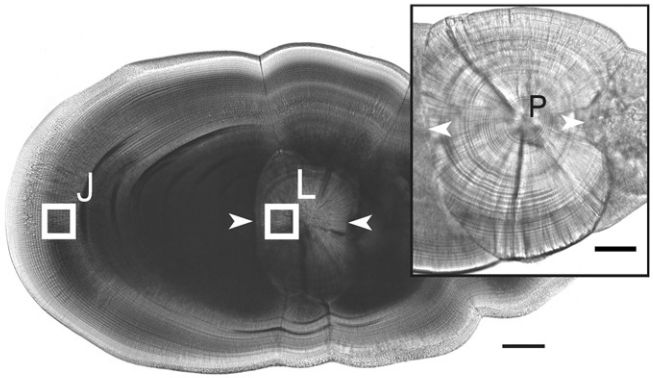

Fig. 2. Images of sandeel juvenile sagittae depicting the landmarks associated with transition from larvae to juvenile (white arrows: first appearance of the accessory primordium). Main photo: position of the laser ablated sites (white squares) selected to represent the larval (L) and juvenile (J) life stages. Scale bar = 100 μm. Inset: close-up view of the larval region showing circular growth emanating from the primordium (P). Scale bar = 50 μm.

settlement (i.e. the July–August period) were identified using an age-radius relationship derived from the subsample of aged individuals. Ablation pits situated in these two identified regions were selected for further analyses, corresponding to 92 larval and 103 juvenile data points.

2.6. Statistical analyses

2.6.1. Assignation of microchemistry data to life stage

Spatial differences in otolith radius at time of metamorphosis were tested with an ANOVA.

2.6.2. Detecting spatial variation in elemental signature of settled juveniles

Spatial differences in elemental signatures were tested on the settled juvenile region of the otolith with ANOVA for Li, Mg, Cu, Rb, Ba and Pb. Mg and Ba were log transformed, Li, Cu and Rb were square root transformed and the cubic root of Pb were considered to satisfy normality and homoscedasticity assumptions. Since Zn, Sr and Mn did not meet these assumptions, geographic differences were tested with Kruskal-Wallis tests. Spatial differences in the elemental signature of the settled juvenile region of the otolith were graphically represented using a plot of the two first principal components of a Principal Component Analysis (PCA). The ability of the otolith chemical signature of settled juveniles to discriminate between sampling locations was assessed using Random forest (RF) classification (Breiman, 2001). The main advantage of this method is that it makes no assumptions on variable distributions or linear relations between variables (Mercier et al., 2011; Stekhoven and Buhlmann, 2012) and can accommodate continuous as well as ordinal or categorical variables. Therefore, all elements showing spatial variation, whether they met parametric tests' assumptions or not, were used as predictors. In RF, each tree is grown using 2/3 of the available data, the remaining 1/3 of the data not used to grow a particular tree and is used to assess the classification accuracy of each tree. RF classification error is then calculated as an aggregate error from all trees. Similarly, differences in the larval region of the otolith among sampling locations were tested with ANOVAs after log transformation of Mg, Zn and Mn (log (1 + x)) while the square root of Li, Cu and Rb was used. Sr, Ba and Pb did not meet normality and homoscedasticity assumptions and, therefore, Kruskal-Wallis tests were used.

2.6.3. Identifying sources of juveniles

Clustering analysis was performed on the settled juvenile region of the otolith to gain insights into the number of sources of juveniles and the connectivity between the locations sampled in the present study. The clustering method developed by Shi and Horvath (2006) was applied, using RF (a supervised learning method) in an unsupervised

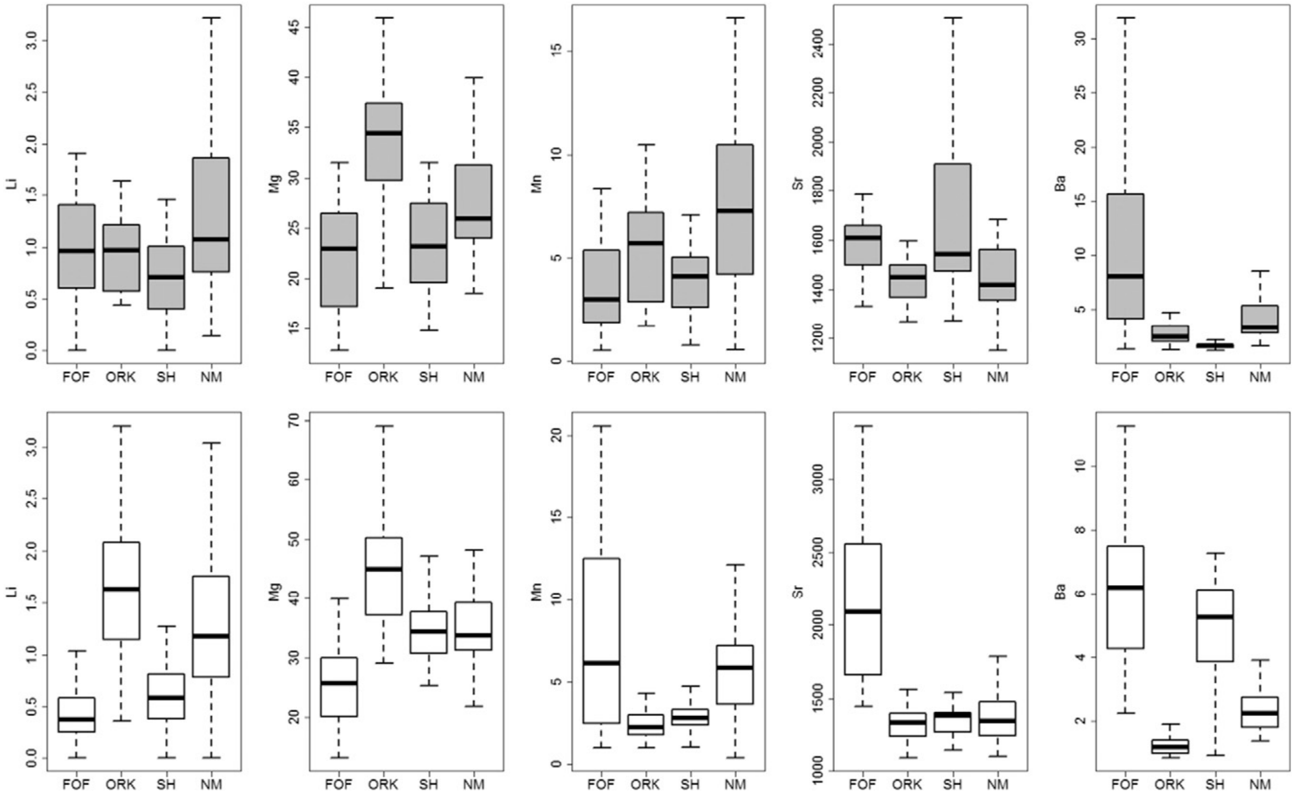

Fig. 3. Boxplots of elemental variations for Li, Mg, Mn, Sr and Ba in the larval (grey) and settled juvenile (white) regions of the otolith. The solid line correspond to the median, the top and bottom of the box are the 75th and 25th percentiles determining the Inter Quartile Range (IQR) while the whiskers extend outside of the box at a distance of 1.5 × IQR.

way, hereafter called RF clustering. RF clustering is a two-step process. In the first step, unsupervised RF was applied to the settled juvenile data set. Capture location (relevant for settled individuals) was ignored in this process; instead, a synthetic dataset was created through random sampling from the product of empirical marginal distributions of the different elements (Shi and Horvath, 2006). Then, RF was used to separate observed from synthetic data (used as the class factor) and to produce a similarity matrix, defined by the frequency at which two individuals end up in the same terminal node of the trees (Breiman, 2001). In the second step, the similarity matrix was transformed into a dissimilarity matrix ($\text{dissimilarity} = \sqrt{(1 - \text{similarity})}$) and used as input for partitioning around medoid (PAM) clustering (Kaufman and Rousseeuw, 1990). The appropriate number of clusters was determined using the gap statistic as defined by Tibshirani et al. (2001).

3. Results

3.1. Assignation of microchemistry data to life stage

Accessory primordia were seen in all sagittae, with oval-shaped otoliths observed even in the smallest fish (45–58 mm), indicating that all were undergoing or had undergone larval metamorphosis to the juvenile stage. Otolith radius at time of metamorphosis was not significantly different among areas ($F_{3,90} = 2.2$, $p = 0.09$). From the subsample of aged individuals, estimated age at metamorphosis was between 44 and 61 days ($N = 31$).

3.2. Spatial variation in elemental signature of settled juveniles

The settled juvenile region of the otolith showed significant geographical variation for Li ($F_{3,98} = 18.9$, $p < 0.0001$, Fig. 3), Mg ($F_{3,98} = 28.7$, $p < 0.0001$, Fig. 3), Rb ($F_{3,98} = 8.02$, $p < 0.0001$), Ba ($F_{3,98} = 97.2$, $p < 0.0001$, Fig. 3), Mn ($H = 22.5$, 3 df, $p < 0.0001$, Fig. 3), Zn ($H = 10.4$, 3 df, $p = 0.01$) and Sr ($H = 55.8$, 3 df, $p < 0.0001$, Fig. 3). Post-hoc comparisons also indicated significant differences between areas, particularly between the Firth of Forth and all other areas (Table 1). Settled juvenile otoliths from the North Minch showed significant differences in five elements to those from Shetland, but exhibited overlap in four elements with otoliths collected in Orkney (Table 1).

From the plot of the two first principal components of a PCA (Fig. 4) it can be seen that Firth of Forth settled juveniles were characterised by high Sr, Ba and Mn signatures, Orkney by high Li and Mg and low Sr and Ba, Shetland by low Li and Sr but high Rb while those from the North Minch on the west coast were characterised by high Li and Zn but low Sr and Ba.

Given that parametric assumptions were not met for a number of elements, RF, which assumes no formal assumptions, allowed for settled individuals to be classified with a high average accuracy (80%) to the location from which they were collected (Table 2). The three locations in the North Sea showed the highest discrimination success, most notably in Orkney with 92.2%, compared to that of the west coast location whose relatively lower classification accuracy suggested a slight degree of overlap in elemental concentrations with other sites.

Table 1
Post-hoc (Tukey's Honest Significant Difference method and pairwise Wilcoxon tests) comparisons performed on the settled juvenile region of the otolith. Area definitions: Orkney (ORK), Firth of Forth (FOF), Shetland (SH) and North Minch (NM). Statistically significant values indicated in bold.

Pairwise comparison	Tukey HSD (adjusted *p*-value)				Pairwise Wilcoxon test (adjusted *p*-value)		
	Li	Mg	Rb	Ba	Mn	Zn	Sr
ORK-FOF	**<0.0001**	**<0.0001**	0.97	**<0.0001**	**0.0026**	0.99	**<0.0001**
SH-FOF	0.59	**<0.0001**	**0.0009**	0.17	**0.0279**	0.16	**<0.0001**
NM-FOF	**<0.0001**	**<0.0001**	0.91	**<0.0001**	0.99	0.96	**<0.0001**
SH-ORK	**<0.0001**	**0.0007**	**0.006**	**<0.0001**	0.40	0.95	0.99
NM-ORK	0.23	**0.001**	0.72	**<0.0001**	**0.003**	0.49	0.99
NM-SH	**0.005**	0.99	**0.0001**	**<0.0001**	**0.01**	**0.025**	0.99

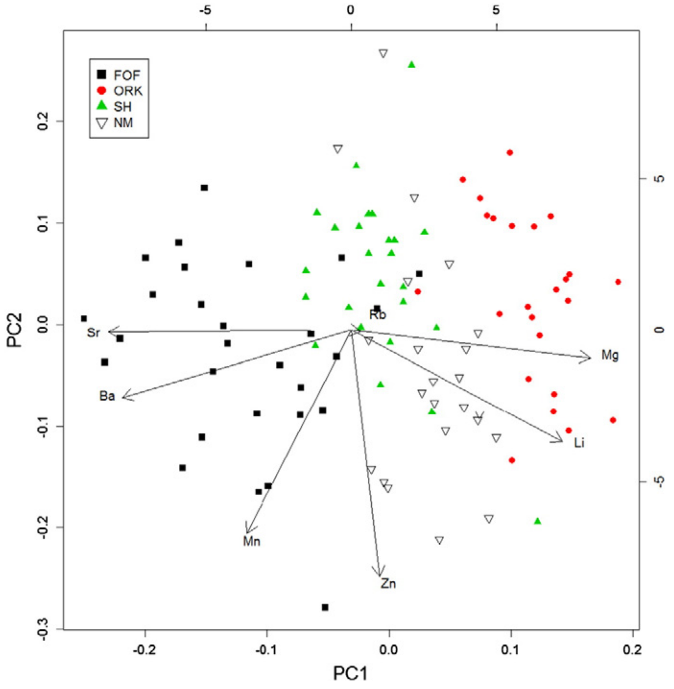

Fig. 4. Plot of the 2 first principal components of a PCA on the settled juvenile region of the otolith.

3.3. Sources of juveniles

Elemental concentrations in the larval region of the otolith showed significant differences among capture locations for Li ($F_{3,88} = 3.5$, $p = 0.018$, Fig. 3), Mg ($F_{3,88} = 12.5$, $p < 0.0001$, Fig. 3), Mn ($F_{3,88} = 5$, $p = 0.003$, Fig. 3), Sr ($H = 20.32$, 3 df, $p = 0.00015$, Fig. 3), Ba ($H = 43.4$, 3 df, $p < 0.0001$, Fig. 3) and Pb ($H = 20.7$, 3 df, $p = 0.0001$), indicating potential differences in their geographic origin. Post-hoc comparisons indeed revealed significant differences in larval elemental concentrations between locations, particularly for Sr and Ba (Table 3). No significant difference was found between the North Minch and Orkney larval signatures, whereas the Firth of Forth differed from other locations across every element except Li (Table 3).

Using these six elements, RF clustering identified 3 clusters of chemically distinct early larval elemental signatures which were all present, albeit at varying levels of prominence, in the four study areas (Fig. 5). The first cluster was the main contributor to Orkney and North Minch sample (Table 4), characterised by high Li and Mg as well as low Sr and Ba (Table 5). The second cluster was the main contributor to Shetland (Table 4), characterised by a low Li and Ba signature (Table 5). The third cluster contributed most to the Firth of Forth sample (Table 4), distinguished by a low Mg and Pb but characteristic high Ba and Sr signature (Table 5).

Table 2
Average classification accuracy (%) obtained for 100 RF performed on independent samples of 20 settled juveniles per sampling location. Area definitions: Firth of Forth (FOF), Orkney (ORK), Shetland (SH) and North Minch (NM).

Location	FOF	ORK	SH	NM
FOF	87.3	0	4.8	7.8
ORK	0	92.2	0.1	7.6
SH	6.5	0.1	74.4	18.9
NM	9.8	9.4	14.6	66.1

Table 3
Post-hoc (Tukey's Honest Significant Difference method and pairwise Wilcoxon tests) comparisons performed on the larval region of the otolith. Area definitions: Orkney (ORK), Firth of Forth (FOF), Shetland (SH) and North Minch (NM). Statistically significant values indicated in bold.

Pairwise comparison	Tukey HSD (adjusted *p*-value)			Pairwise Wilcoxon test (adjusted *p*-value)		
	Li	Mg	Mn	Sr	Ba	Pb
ORK-FOF	0.98	**<0.0001**	0.24	**0.0023**	**0.00071**	**0.0073**
SH-FOF	0.39	0.96	0.99	0.99	**<0.0001**	**0.024**
NM-FOF	0.33	**0.046**	**0.0052**	**0.012**	0.063	**<0.0001**
SH-ORK	0.22	**<0.0001**	0.35	**0.011**	**0.0072**	0.99
NM-ORK	0.60	0.057	0.43	0.99	0.077	0.99
NM-SH	**0.01**	0.12	**0.0096**	**0.022**	**<0.0001**	0.99

Table 4
Contributions of the different larval clusters (in %) to the samples (RF clustering). Area definitions: Firth of Forth (FOF), Orkney (ORK), Shetland (SH) and North Minch (NM).

Location/cluster	1	2	3
FOF	8.0	12.0	80.0
ORK	76.2	19.0	4.8
SH	11.1	74.1	14.8
NM	63.2	26.3	10.5

4. Discussion

The presence and scale of significant geographical variation in juvenile otolith chemistry among settled locations (segregated from each other by 100–400 km) is consistent with previous studies in this region (Gibb et al., 2007; Tobin et al., 2010; Wright et al., 2006, 2010) and fits well with the evidence of restricted horizontal movements following settlement (Gauld, 1990; Kunzlik et al., 1986). Relative concentrations of certain elements were similar to those found in juvenile gadoids sampled in the same areas. Elevated levels of Sr in the Firth of Forth were also found in juvenile haddock (Wright et al., 2010). The relatively low level of Mn seen in the Shetland otoliths was consistent with that found in 0-group cod (Wright et al., 2006; Gibb et al., 2007), haddock (Wright et al., 2010) and whiting (Tobin et al., 2010). The elevated levels of Mn seen in the North Minch have also been found for juvenile whiting (Tobin et al., 2010), cod (Wright et al., 2006) and haddock (Wright et al., 2010). Although we cannot explain the observed differences in elemental signatures, variation in water elemental concentrations have been reported over the spatial scales we found (e.g. Balls et al., 1993) and there are salinity and temperature gradients (Berx and Hughes, 2009) that may potentially modify some elements (Bath et al., 2000; Martin and Thorrold, 2005; Miller, 2011) important to the elemental signatures.

The apparent differences between the larval and juvenile elemental signatures may be related to the increased physiological stress associated with metamorphosis on certain elemental concentrations, as found in other studies (de Pontual et al., 2003; Arai et al., 1997; Toole et al., 1993). While Mg concentrations showed similar trends in both larvae and juveniles across our study areas, we found consistently higher concentrations of this element in the settled juveniles, seen also in *A. tobianus* (Laugier et al., 2015). The highly significant differences seen in the juvenile otoliths among all four study areas may relate to the sandeel's habit of burying in sand following settlement (Wright et al., 2000). Differential anoxic conditions of water overlying the sediment may explain these differences, as hypoxia is known to affect elemental

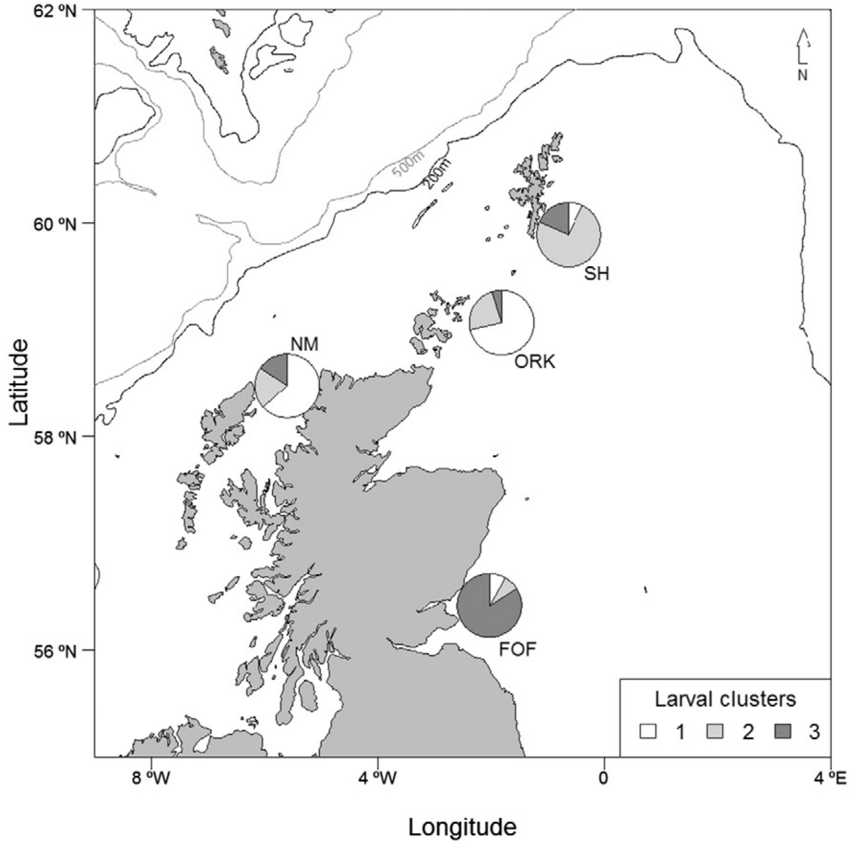

Fig. 5. Chart of the sampling locations and pie charts representing the contribution of the three juvenile sources identified through RF clustering. Area definitions: Firth of Forth (FOF), Orkney (ORK), Shetland (SH) and North Minch (NM).

Table 5
Mean elemental signature of the 3 larval clusters identified by RF clustering, standard deviation is indicated between brackets.

Cluster	Li	Mg	Mn	Sr	Ba	Pb
1	1.25(0.69)	33.3(8.49)	7.2(3.06)	1388.3(103.87)	3.63(2.26)	0.09(0.08)
2	0.64(0.41)	23.57(5.12)	4.54(3.13)	1660.4(298.41)	2.15(0.98)	0.09(0.13)
3	1.05(0.63)	23.09(6.38)	3.74(3.97)	1734.41(379.46)	9.71(8.62)	0.02(0.01)

uptake (e.g. Limburg et al., 2015) through changes in metabolism linked to feeding, growth, food supply and swimming (Behrens and Steffensen, 2007; Petersen and Pihl, 1995).

The unsupervised learning method used to cluster larval elemental signature appears well suited to studies of propagule dispersal where no representative baseline sample is available to identify the origin of collected samples. The clustering approach does not directly provide information on the location of the different larval sources but is able to infer the number of natal sources contributing to a settlement site. For example, in the Adriatic Sea, clustering analysis revealed that mackerel adults originated from a single juvenile source (Papetti et al., 2013) and from 3 juvenile sources in white sea bream with variable levels of connectivity between sites (Di Franco et al., 2012). Although a supervised approach is preferable, the difficulty of obtaining a sufficiently representative baseline is often prohibitively expensive. For example, to have obtained baseline samples for *A. marinus* would have required repeated, large scale dedicated ichthyoplankton surveys due to the variation in hatch times of this species (Wright and Bailey, 1996). Although both supervised and unsupervised approaches can be confounded by the presence of an external source of larvae sharing a similar elemental signal with a source from the studied area, the clustering approach is able to identify the contribution of an external source, provided its elemental signature is distinct. Some previous analyses of fish dispersal using otolith microchemistry have used parametric models that require elemental concentrations to follow multivariate normal distributions (e.g. White et al., 2008). However, the RF clustering approach adopted in this study relaxes both the requirements of continuous multivariate data and the distributional assumptions (Shi and Horvath, 2006) as in the tree building process, at each node, classification results from the binary splitting of predictor variables. This method handles categorical, as well as ordinal and continuous data, including highly skewed and truncated distributions, common in microchemistry analyses (Higgins et al., 2013). It is therefore readily applied to any otolith microchemistry data set.

Only three larval clusters were identified in this study and the composition differed with respect to elements found to be important to the separation of settled juvenile elemental signatures (e.g. Mg, Ba, Sr on axis 1 PCA; Fig. 4). Assuming these clusters reflect different natal sources we can get some indication of the spatial scale of separation based on the relative contribution to sample locations (Di Franco et al., 2012; Calò et al., 2016). However, given that the location of the natal source is not known we cannot be sure to what extent clusters reflect distinct natal sources. Although all three clusters contributed to all four sample locations there were major regional differences. Most Firth of Forth juveniles had a cluster 3 signal associated with low Mg, high Ba and high Sr. The low proportion of individuals assigned to cluster 3 in other sample locations and particularly in the closest sample location, Orkney, is suggestive of a largely separate natal source and might result from a small overlap in the signature of the natal sources. Biophysical models also suggest that larvae in the Firth of Forth may receive little input from the large Orkney spawning area further north, and circulation in the Firth of Forth region is expected to lead to a high degree of retention (Christensen et al., 2008; Gallego et al., 2004; Proctor et al., 1998). Further support for isolation between the Firth of Forth and sandeel aggregations further north come from differences in recruitment and the breeding success of sandeel-dependent predators (Frederiksen et al., 2005) between the Northern Isles and Firth of Forth. It is possible that grounds to the south and east of the Firth of Forth may have been a larval source, as these areas were not sampled in this study. However, this is unlikely given the relative geographical and hydrographical isolation indicated by biophysical models (Christensen et al., 2008; Proctor et al., 1998).

The similarity in larval chemistry between the North Minch and Orkney sites, as indicated from the same dominant cluster and lack of pairwise difference in element concentration can either be explained by a common larval source or a limited difference in the site specific elemental signature. Similarity in larval signature may be a result of a shared water-column driven signature at the time of hatch (e.g. Ashford et al., 2008). The Orkney sample site is part of the most extensive and productive spawning grounds in the study area and the North Minch sample site is well connected hydrographically to this area through largely wind-driven circulation patterns at the time of hatching (Turrell et al., 1992; Winther and Johannessen, 2006). Hence the local currents may homogenise the signature of the North Minch and Orkney so that it appears as a single source.

The dominance of a different larval cluster for Shetland from that of Orkney and North Minch appears consistent with past evidence on larval distribution for a separate natal source (Wright and Bailey, 1996). The Shetland site corresponds to the smallest sandeel ground investigated, and evidence from pre-settled juvenile distributions, changes in juvenile hatch date composition (Wright, 1996) and a biophysical model (Proctor et al., 1998) suggests that larval export to this area or immigration is important to sustaining this ground. The difference between the juvenile and larval signature from this site relative to other sites seems consistent with changes in or mixing of natal composition of the settled juveniles.

Since 2010, management of sandeel in the North Sea has moved from a single stock assessment to seven separate sub-components (ICES, 2010), based on evidence from distribution, predicted larval transport, demographic variation and differences in regional dynamics (Jensen et al., 2011; Boulcott et al., 2007; Christensen et al., 2008; Pedersen et al., 1999; Proctor et al., 1998). Although we are only able to infer probable scales of mixing from our study, the results do appear consistent with previous evidence for limited connectivity between the Firth of Forth aggregation and the northern aggregations examined (Proctor et al., 1998). Based on the larval elemental signatures, the ncMPA locations in north-west Orkney and southern Shetland appear to have different natal sources, although the composition of Shetland juveniles is consistent with past evidence for immigration into this area (Wright, 1996). Clearly, further work is required to identify the natal sources and to assess the impact of environmental change on the distance and direction of larval dispersal, which will inform the relevant positioning of future MPAs designed to conserve sandeel sub-populations. The random forest clustering approach applied here could be further developed by the inclusion of a few targeted larval samples that would help identify the geographic location of larval clusters.

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