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Evaluating and advancing methodologies to validate the origin of capture of fish: Stable Isotope Technologies (MMO1192)



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MMO1192: Evaluating and advancing methodologies to validate the origin of capture of fish: Stable Isotope technologies

September 2019



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Executive Summary

The isotopic composition of the tissues of marine animals varies reflecting geographic differences in the isotopic composition of their diets and/or the physico-chemical nature of the surrounding water. If these spatial variations are known or can be predicted, then tissue isotopic compositions can be used to infer the spatial origin of an animal.

The environmental drivers underpinning spatial variation in stable isotopes in the marine environment are relatively well-understood and are linked to commonly observed physical and biological parameters. Consequently, geographic variations in isotopic compositions can be predicted via spatial statistical modelling. This is a major advantage of stable isotope approaches over alternative natural tracers for traceability studies.

Isotope-based traceability can be performed by comparison of the isotopic composition of the test samples and either a reference dataset drawn from known discrete origins or a continuous assignment method based on comparison to spatial statistical models of isotopic variation (isoscapes).

While still in development, the combined stable isotope compositions of carbon (C) nitrogen (N) and sulphur (S) shows great promise as a geographic indicator for marine food stuffs using both discrete and continuous assignment approaches. On the scale of ocean basins, stable isotope-based traceability may match or exceed genetic methods in accuracy and precision. An understanding of the limitations in terms of accuracy and precision is essential for best use of this tracer technology.

In a UK context, C N and S isoscapes have been built for the entire UK shelf, and initial reference datasets are being compiled for major cod fishery locations across the North East Atlantic (FAO ¹27) Area.

The major limitations of stable isotope based geographic assignment in general are the relatively small number of available isotope tracers (usually 2 or 3) and the potential for non-spatial (i.e. behavioural) influences on tissue isotopic compositions. In a UK context, the major limitation is the lack of reference datasets of the isotopic composition of known origin fish.

We assessed the level of implementation of isotopic analysis for each species of interest against the Technology Readiness Levels (TRL) as defined by the European Research Council (2014) (Table 1): the higher the number meaning the more advanced and operationally applicable.

¹ United Nations Food and Agriculture Organisation (UN FAO)

Table 1 - Technology Readiness Levels

Level	Description
TRL 1	Basic principles observed
TRL 2	Technology concept formulated
TRL 3	Experimental proof of concept
TRL 4	Technology validated in lab
TRL 5	Technology validated in relevant environment (industrially relevant environment in the case of key enabling technologies)
TRL 6	Technology demonstrated in relevant environment (industrially relevant environment in the case of key enabling technologies)
TRL 7	System prototype demonstration in operational environment
TRL 8	System complete and qualified
TRL 9	Actual system proven in operational environment (competitive manufacturing in the case of key enabling technologies; or in space)

Table 2 - Summary table of current Technology Readiness Levels (TRL) of isotopic analysis tools available for the species under focus in this study

Fishery product (common name)	Assignment technique	Resolution	TRL	TRL Description
Cod	Discrete	Sub-ICES area	7	System prototype demonstration in operational environment
Plaice	Discrete	Regional	5	Technology validated in relevant environment
Dab	Discrete	Regional	5	Technology validated in relevant environment
Whiting	Discrete	Poor	1	Basic principles observed
Gurnard	Discrete	Regional	5	Technology validated in relevant environment
Long Rough Dab	Discrete	ICES area	3	Experimental proof of concept
Anglerfish	Discrete	Regional	5	Technology validated in relevant environment
Haddock	Discrete	Regional	5	Technology validated in relevant environment

Lemon Sole	Discrete	Poor	1	Basic principles observed
Herring	Continuous	Sub-ICES area	7	System prototype demonstration in operational environment
Nephrops	Continuous	Regional	7	System prototype demonstration in operational environment
Anglerfish	Continuous	Poor	3	Experimental proof of concept
Sole	Continuous	Regional	3	Experimental proof of concept

The TRL for stable isotope methods will be best advanced through the development and maintenance of reference datasets particularly assessing species-specific isotopic variation across key locations.

Integration of stable isotope and other natural tracers is likely to improve the potential for traceability above that provided by any method in isolation.

1. Introduction

As part of the Marine Management Organisation's (MMO) ongoing work looking at enhancing provenance and traceability the MMO sought an assessment of methods for confirming where fish sold ashore were originally caught to potentially support and verify other systems for managing compliance and enforcement. This report concerns stable isotopes, one of four technique classes previously identified as promising by the MMO along with fatty acids, trace elements and genetics.

This report synthesises key recent developments regarding spatial distribution, biological control and study design protocols associated with stable isotope based traceability of fish products, their impacts on the practicalities of using stable isotopes as geographic tracers, and our level of knowledge particularly in the context of UK marine foodstuffs. The report consists of the following:

- Section 2 reviews our understanding of stable isotope variability in the environment and the practical methods associated with isotope-based traceability.
- Section 3 provides an assessment of the potential accuracy and precision available using stable isotope-based traceability approaches in a UK context. This section draws on currently available datasets and also identifies knowledge gaps limiting the technological readiness level of the method
- Section 4 provides a personal view on research needed to enhance the technological readiness level of the stable isotope method as a tracer of geographic origin in the UK marine foodchain.

The main advantage of using stable isotopes as geographic tracers is the predictable nature of spatial variation in stable isotope compositions within marine ecosystems.

Geographic traceability largely proceeds by comparing the chemical composition of a tissue of unknown origin to some database of compositions expected in discrete regions. The environmental and biogeochemical factors responsible for producing spatial gradients in stable isotope compositions of carbon (C) nitrogen (N) oxygen (O) and sulphur (S) in marine environments are relatively well understood and have been reviewed recently (e.g. Trueman and St John Glew 2019). Parameters such as water temperature, water depth and mixing, chlorophyll concentration and nitrate concentration capture most of the spatial variance in isotopic compositions, and most of these variables are commonly observed across UK and NE Atlantic shelf seas. Accurate and precise models of the spatial variation of stable isotopes of C, N and S in marine food chains across the UK shelf seas have been developed (St John Glew et al., 2019) and are suitable for both desk-based viability studies and for assignment of unknown origin samples.

The practical value of stable isotope-based methods for traceability studies will improve with the assembly of large, spatially referenced databases of isotopic compositions of fishes (and invertebrates) of interest.

2. Biogeochemical context

2.1 Stable isotopes

Isotopes are forms of the same element with different numbers of neutrons, and stable isotopes are isotopes that are not radioactive. The difference in mass between two isotopes of the same element creates small differences in the rates of reactions and strengths of chemical bonds. Isotopes can therefore be separated from one another during chemical reactions (termed 'discrimination'). Elements with low atomic numbers are relatively light so that the differences in mass between two isotopes is a larger fraction of the total mass of the element, leading to larger isotopic discrimination. Light elements such as C, N and S commonly show relatively large levels of isotopic discrimination relative to measurement errors. This report largely focuses on stable isotopes of carbon nitrogen and sulphur, as these three elements show the most practical promise for traceability within marine systems.

Isotope ratios vary spatially, at varying scales, across terrestrial, aquatic and marine landscapes as spatial differences in the hydrological cycle, nutrient cycling and biological processes exert effects on isotopic abundance (Bowen, 2010). Pronounced and predictable isotopic variations in hydrogen and oxygen in precipitation, and geologically-based variations in strontium (Sr) isotopes are extensively used in traceability and forensic approaches in terrestrial settings (Bowen, 2010, Meyer-Augenstein, Chesson). Seawater is essentially homogenous with respect to isotopic compositions of O, H and Sr - at least in a forensic traceability context. However, in marine systems, *biological processes* associated with nutrient fixation into primary production create spatial variations in carbon, nitrogen and sulphur isotopic compositions of primary production that are passed through food chains and can be used to study spatial questions in marine animals (Jaeger et al., 2010).

2.2 Spatial variation in carbon isotopes

The isotopic ratio of carbon in primary producers is determined by the isotopic composition of the carbon source and the extent of isotopic fractionation (change in isotopic ratio) during photosynthesis. The amount of fractionation of carbon isotopes during photosynthetic fixation by marine phytoplankton is influenced by temperature, phytoplankton type and growth rate. At the scale of ocean basins, carbon isotopic compositions of phytoplankton largely track spatial temperature gradients (Hofmann et al., 2000). In shelf and coastal seas carbon isotope compositions of plankton may be additionally influenced by carbon inputs from terrestrial sources, macroalgae and/or seagrasses. Benthic and pelagic food chains in the same spatial location may differ in carbon isotope compositions largely due to isotopic discrimination processes during transformation of organic carbon in the water column and at the sediment-water interface.

2.3 Spatial variation in nitrogen isotopes

The isotopic ratio of nitrogen at the base of the food chain is influenced primarily by the type and isotopic composition of the nitrogen source fixed by phytoplankton. Nitrogen uptake and isotopic composition is in turn controlled by the biogeochemical

nature of the ambient water column, such as the presence of nitrogen fixing bacteria, the concentration of nitrates and the strength of upwelling or down-welling waters (Montoya, 2007, Trueman et al., 2012a). The processes underpinning spatial variability in the nitrogen isotope composition of plankton are complex and relatively difficult to predict, but at a global scale, the relative isotopic composition of phytoplankton co-varies with the relative concentrations of nitrate, CO₂ and phosphorus in surface waters (Ryabenko 2013). In coastal and shelf regions, spatial variations in the stable isotope composition of phytoplankton and other organisms may be additionally influenced by anthropogenic sources, especially sewage and agricultural fertilisers, and by the degree of water column mixing. The isotopic composition of nitrogen in animals is altered through food chains as the lighter isotope is preferentially excreted, so that higher trophic level animals build tissues with higher proportions of ¹⁵N. Benthic food webs are similarly enriched in ¹⁵N compared to pelagic food chains due to preferential loss of ¹⁴N via microbial processing of organic nitrogen.

2.4 Spatial variation in sulphur isotopes

The isotopic ratio of sulphur is more constant in open ocean environments, but strong gradients have been observed between terrestrial and marine ecosystems (Fry, 2002). In coastal and shelf sea environments, further factors complicate spatial stable isotopic variation resulting in isotopic gradients at smaller scales than typically observed within open ocean settings. Sulphur isotope variability in ocean waters is rather less understood, and only recently included in spatial investigations of fully marine ecosystems.

2.5 Spatial assignment methods and isoscapes

Within coastal environments, a greater range of isotopically distinct primary producers are present than found within the open ocean and terrestrial run off and pollution can also influence isotopic composition, with sewage, agricultural and fisheries farm waste inputs relatively enriched in ¹⁵N and ¹³C (Montoya, 2007). In addition, water column depth can have a significant influence on isotopic composition, due to varying degrees of mixing and re-suspension of sedimented organic matter. These strong isotopic gradients, across small spatial scales, mean shelf sea environments serve as ideal locations for isoscape modelling and subsequent isotopic assignments. However, high levels of isotopic variability in space and time can also lead to unpredictability, limiting the value of isotope-based traceability in highly dynamic regions.

Stable isotope based geolocation has become a standard method in terrestrial ecology and forensics largely because of the relatively predictable nature of spatial gradients in isotopic compositions across space. By mapping isotopic gradients in two or more dimensions, a continuous surface model of isotopic variability can be generated. Such models or isoscapes, allow the likely origin of a sample to be estimated with no prior information. An isoscape can be a mechanistic or statistical spatial model of the distribution of isotope ratios across space (Bowen, 2010). Isoscapes have previously been used to determine origin of animals (Hobson et al., 1999), plants (West et al., 2006) and humans (Ehleringer et al., 2008) and to determine traceability within forensic, food and consumer goods contexts, in a

terrestrial environment (Chesson et al., 2010). The use of isoscapes in marine settings has lagged behind terrestrial applications, largely due to the increased practical complexity of obtaining reference samples at sufficient spatial and temporal resolution. In the past 10 years significant progress has been made in constructing marine isoscapes for carbon and nitrogen both from statistical interpolation from geo-referenced samples and from mechanistic models (Trueman and St John Glew 2019).

2.6 Isotopic assignment – discrete and continuous approaches

Isotope-based traceability works by comparing the isotopic composition of an animal's tissue with some spatially referenced comparative dataset. Such reference datasets may be compiled from tissues of the same species or from other organisms. Reference datasets constructed from the same organism (e.g. a cod reference dataset) may provide more accurate and precise geo-location. The isotopic composition of an animal's tissue reflects both underlying spatial gradients in isotopic compositions of primary production, and isotopic variability associated with diet and trophic level. Consequently, reference datasets compiled from other organisms (or different sized organisms in size-based food webs) need to be calibrated to account for dietary, behavioural and/or trophic level effects. Calibration introduces more uncertainty and error, but means that multiple species can be compared to a single reference dataset. The choice of reference datasets will depend on the accuracy and precision requirements of the question, practical availability of suitable reference samples and the spatial gradient in isotopic compositions expected in the region under study.

Assignment may be performed by determining the relative probability of origin among a specified set of possible locations (discrete assignment) or by assigning to a possible region with no prior knowledge of potential areas (continuous assignment).

The distinction between continuous and discrete assignment is a critical aspect of study design for traceability. Discrete assignment depends on having a reference dataset or 'fingerprint' that can characterise ALL possible or likely sources. Discrete assignment methods are ideal where the variable or variables acting as tracers vary uniquely between the discrete locations under study. If spatial variation in tracers does not coincide with the regions under study, discrete assignment methods are less likely to be effective. Continuous assignment, by contrast, draws on reference spatial models (isoscapes) to determine the most likely spatial origins across a continuous map surface. Continuous surface assignments may yield lower apparent accuracy/precision results because all spatial areas are treated as possible. Currently only stable isotope-based traceability methods allow full continuous assignment within UK shelf seas.

2.7 Discrete assignment

Discrete assignments probabilistically geolocate an animal to the most likely of a number of predetermined regions. Discrete assignment therefore requires a reference dataset of expected isotope values in each of the potential source areas. Discrete assignment explicitly assumes that all possible source areas are contained

within the reference datasets. Because the discrete assignment approach makes an explicit prior assumption that there are a limited number of possible source regions, discrete assignment is less demanding in terms of reference databases, and typically produces higher apparent accuracy and precision. However, the true accuracy and precision for any discrete-assignment based approach will be influenced by the degree to which the reference datasets adequately capture the true variance within the grouping region, and the possibility of sources other than those contained in the reference dataset. Most natural tracer-based geo-assignment methods such as trace element, genetic and fatty acid based approaches typically use discrete assignment approaches.

Discrete assignment approaches may be further divided into those where the spatial range of the reference populations are decided based on management priorities (i.e. assigning among ICES areas) or where the potential spatial populations are decided based on the known isotopic variability within the region. In the literature, discrete assignments have been used to discriminate between different populations, stocks and nursery grounds in marine fish, invertebrates and mammals (Rooker et al., 2008, Vander Zanden et al., 2015). Discrete assignment can be performed using approaches such as linear or quadratic discriminant analysis, or through comparison with distributions of reference datasets using Bayesian logic.

2.8 Continuous assignment

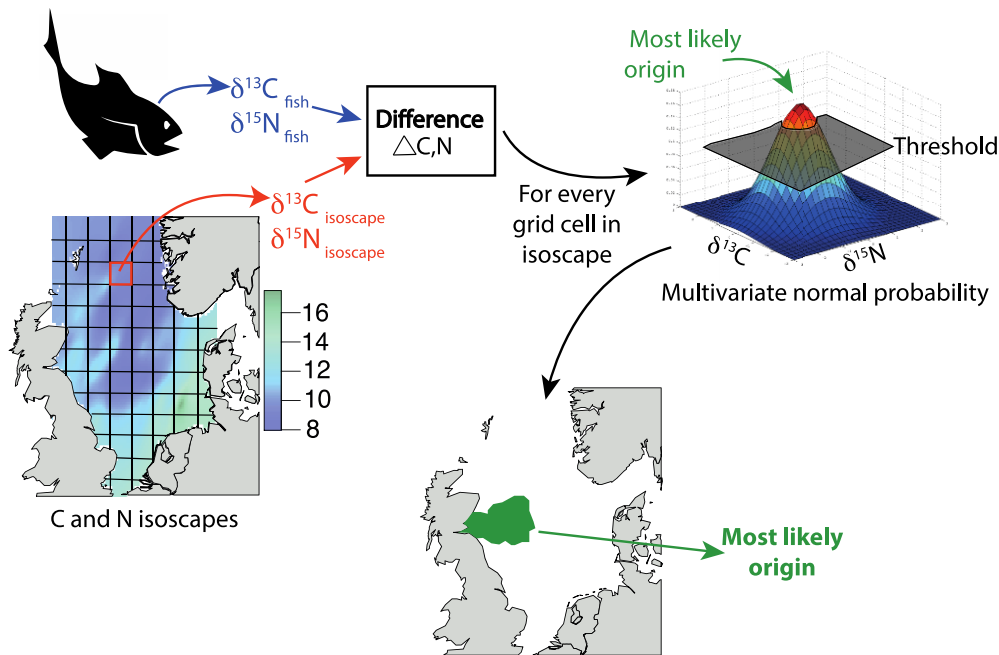
In the continuous assignment approach, the isotopic composition of the sample is compared to that predicted for every cell within the isoscape, and the difference expressed as a probability of origin usually scaled over the isoscape region (Vander Zanden et al., 2015, Trueman et al., 2017). The likelihood of origin associated with each cell is a function of the isotopic difference between the sample and cell and the uncertainty associated with the isotopic values both of the measured sample and the predicted isoscape (Figure 3). The theory of propagation of uncertainty in continuous assignment approaches is relatively well advanced (Wunder 2010, Trueman et al. 2017).

Irrespective of the assignment method used, the isotopic composition of an animal's tissues reflects the isotopic composition of its diet. This means that behavioural variations other than spatial factors can influence an animal's isotopic composition. For instance in fish, diet and trophic level is strongly linked to body size, so variations in body size will influence the isotopic compositions of tissues in animals sharing a common location. Diet variability among individuals may also influence isotopic compositions, especially in complex coastal or estuarine locations. Good use of isotope-based geolocation requires that behavioural influences on tissue isotopic compositions are acknowledged and, where possible quantified, for the species in question. At present this is a major limitation in the technological readiness level for isotope-based geolocation.

Time is also a consideration for tracer-based geoassignment. If the assignment individual migrates across the isotopic gradients at a faster pace than nutrients are metabolized and tissues are formed, assignment of that individual will not be possible. The ability to use natural tracer based methods therefore also depends on

the relative rates of tissue growth and migration across spatial chemical gradients (McMahon et al., 2013). When geolocating a predator to its foraging location using carbon and nitrogen isotopes, prey are usually assumed to be static, however this is rarely true in the marine environment. **Tracer based approaches therefore require a reasonable degree of expert knowledge to determine when they are appropriate.** We expand on a few of the considerations below:

Figure 1 - Schematic illustrating continuous isoscape – based assignment methodology



2.9 Complications with continuous assignment

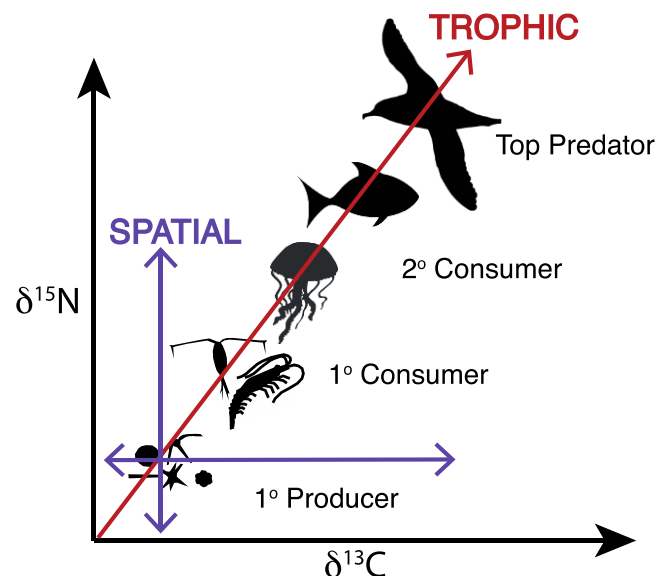
Isoscape models are based on numerous assumptions and uncertainties including baseline organism physiological differences, temporal and spatial variability, spatial interpolation errors and the spatial resolution of the data. The reliability of an isoscape model and its utility across multiple applications, is dependent on the ability to quantify these variance-generating processes across the geographic region of interest.

For organic isoscapes, isotope samples need to be taken from tissues of organisms at even spatial resolutions across the region of interest; the chosen animal is termed the baseline organism. However, the isotopic composition of both carbon and nitrogen changes throughout the food web due to isotopic fractionation (trophic discrimination), occurring at each stage along the biological pathway. Consumers are typically reported to be enriched by 3.0-3.4‰ in $\delta^{15}\text{N}$ (Minagawa and Wada, 1984), and $\sim 1.0\%$ in $\delta^{13}\text{C}$ (Peterson and Fry, 1987) in comparison to their prey (Figure 4). As baseline organisms used to construct marine isoscapes, are often not the same species as the assignment individual, as in the UK Shelf Seas, trophic discrimination and variability between values needs to be considered when producing and using organic marine isoscapes. Ideally, known-origin samples of test species and the organism used to build reference datasets or isoscapes would be

available to quantify species effects on isotopic calibration (and the associated uncertainty). Where that is not possible, estimates of trophic separation can be made from literature-based predictions of species' trophic level and assumed isotopic trophic discrimination. In size structured marine food webs, an additional correction for body size effects on trophic level may increase assignment accuracy, but body size data may not be available for all potential test samples (for instance if testing a processed food product). In any case, uncertainty associated with isotopic calibration should be estimated and explicitly considered in any assignment (e.g. Trueman et al. 2017).

As noted above, the time taken to assimilate diet isotope values into the tissues being analysed (termed 'turnover rates') needs to be considered, particularly in the context of migratory animals. Isotopic assignment will be problematic where turnover rates are slow in relation to the movements of the animal (for example in a highly migratory fish with a low metabolic or tissue growth rates such as adult spurdog).

Figure 2 - Schematic demonstrating trophic enrichment of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in a marine food web



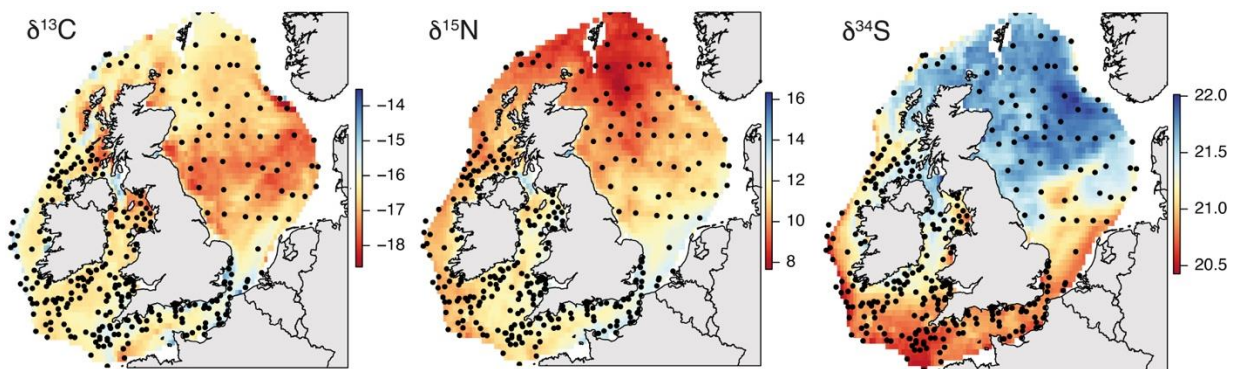
Marine nutrient availability and photosynthesis rate fluctuates over seasonal, yearly and longer time scales, influencing absolute $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ ratios and their potential spatial distributions. In some marine environments isotope ratios remain relatively stable, for example North Sea broad scale isotopic patterns have remained relatively consistent over decadal to centennial time periods (MacKenzie et al., 2014, Trueman et al., 2017), likely due to the dominant influence of static environmental variables on isotopic composition within this area (MacKenzie et al., 2014). However, some more dynamic regions with strong terrestrial inputs, such as the Irish Sea and eastern English Channel may be more susceptible to temporal variations in isotopic ratio. As with any natural tracer, understanding the temporal stability of regional isotopic differences is important. In a UK context, sample-based models of the spatial variations in carbon and nitrogen isotopes within the North Sea have been constructed based on samples separately collected in 2001, 2011 and 2015.

2.10 Continuous isoscapes in a UK context

The North Sea, and UK shelf seas in general, are among the best characterised marine regions globally in terms of spatial and temporal variations in stable isotope compositions.

To date, carbon, nitrogen and sulphur continuous spatial isoscape models have been constructed across the UK Shelf Seas (St. John Glew et al., 2019) (Figure 1). To construct isoscape models of UK shelf seas, 627 jellyfish samples of 7 different species were collected from 308 stations across the UK shelf seas between August 2015 and December 2016. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ isoscapes were predicted using a Bayesian hierarchical modelling approach using integrated nested Laplace approximations (INLA) via the R-INLA package. Satellite and modelled environmental data, averaged over the two sample years, were also incorporated into the prediction models.

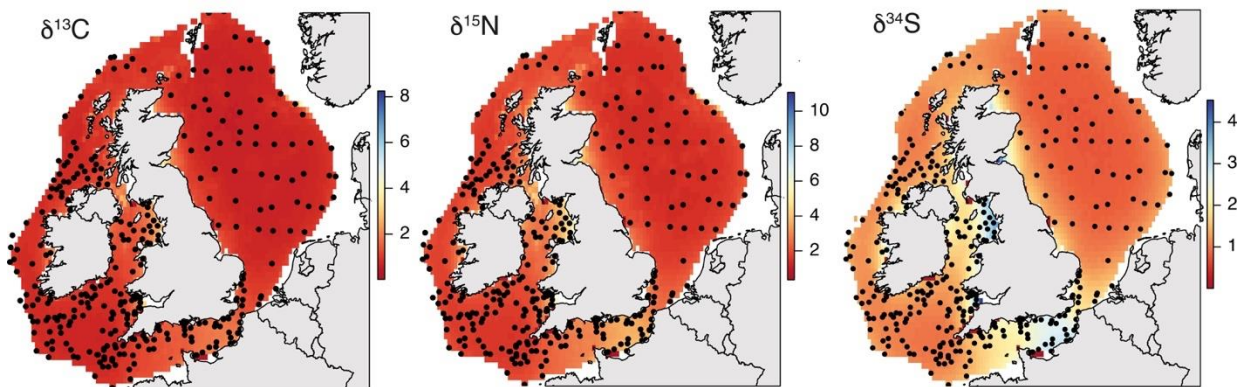
Figure 3 - Carbon, nitrogen and sulphur isoscape models for the UK shelf seas. Values based on 7 species of jellyfish sampled between August 2015 and December 2016. Filled circles represent sampling locations (St. John Glew et al., 2019).



These models are, at present, the most spatially extensive sample-based marine isoscapes anywhere in the world.

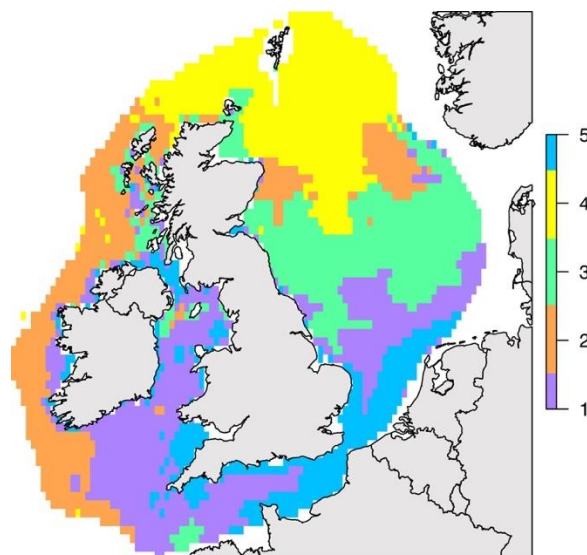
Variance surfaces of the prediction model of UK Shelf Sea isoscapes for carbon, nitrogen and sulphur have also been built (St. John Glew et al., 2019)(Figure 5), showing the relative uncertainty surrounding the isoscape predictions across space. Quantifying the relative uncertainty associated with a tracer among regions is essential for accurate and reliable traceability, whether using a stable isotope or alternative approach to traceability. We have explicitly quantified spatial uncertainty associated with our models of stable isotope compositions across the entire UK shelf sea. We are therefore able to highlight areas of the UK Shelf Seas with relatively certain and fixed isotope values (i.e. central North Sea, Celtic Sea), and those that are likely to be more dynamic and more difficult to assign individuals to (i.e. eastern English Channel and eastern Irish Sea).

Figure 4 - Variance surfaces of the modelled carbon, nitrogen and sulphur isoscapes (St. John Glew et al., 2019). Areas of relatively high variance indicate areas where isotope-based assignment will be less precise/accurate



Using the isoscape models, it is possible to illustrate regions within the UK shelf seas that are expected to be isotopically similar using clustering statistical approaches. The ability to predict a-priori whether animal products are expected to vary chemically between or among regions is a major advantage in isotope-based methods for traceability. In Figure 5 k-means cluster analysis is used to group regions according to similarity in the C, N and S isoscape models. This provides an initial estimate of the likely spatial scales and regional patterning underpinning stable-isotope based traceability in a UK shelf sea context.

Figure 5 - Example of how UK Shelf can be divided into 5 discrete regions displaying similar carbon, nitrogen and sulphur isotope ratios. Illustration of regions likely to be isotopically distinctive



3. Case studies

3.1 Assignment accuracy and precision

Accuracy and precision are critical metrics in applied traceability studies, but estimating accuracy and precision associated with natural tracer-based spatial assignment is not simple, and commonly poorly resolved (Trueman et al., 2017). Assignment accuracy is defined as the proportion of 'correct' assignments, and assignment precision is defined as the size of the area that individuals are assigned into. Accuracy and precision associated with natural tracer geo-assignment methods varies across space with the natural variability and predictability of the tracer, and among species as behavioural traits of the animals vary. For instance, spatial precision and accuracy will be maximized in sessile organisms distributed between regions with contrasting isotopic compositions, and will be minimized in highly migratory animals travelling across small isotopic gradients. There will also always be a trade-off between accuracy and precision, with more precise assignments to smaller areas of the isoscape achieving lower accuracy. If highly precise assignments are not required, and individuals can be assigned into larger management areas, such as sea areas, higher accuracies are more achievable.

Accuracy and precision is best evaluated by assigning tissues of animals of known origin either among specified discrete regions or, in a continuous assignment approach, into increasingly small geographic regions (Trueman et al. 2017). **In either case datasets of known origin samples are needed, and these are typically missing, especially in a UK shelf seas context.**

3.2 Ocean basin-scale assignment: Atlantic cod case study

To illustrate the potential of using isotope methods to discriminate among regions, a database of the stable isotope (CNS) compositions of cod (*Gadus morhua*) muscle has been assembled, collected from fisheries research surveys across major fishery locations within the north east Atlantic (Figure 6). Icelandic and Norwegian / Barents Sea fish are to be added in the near future. Data are plotted in Figures 7 and 8.

Figure 6 - Sampling regions for regional discrimination test for *G. morhua*

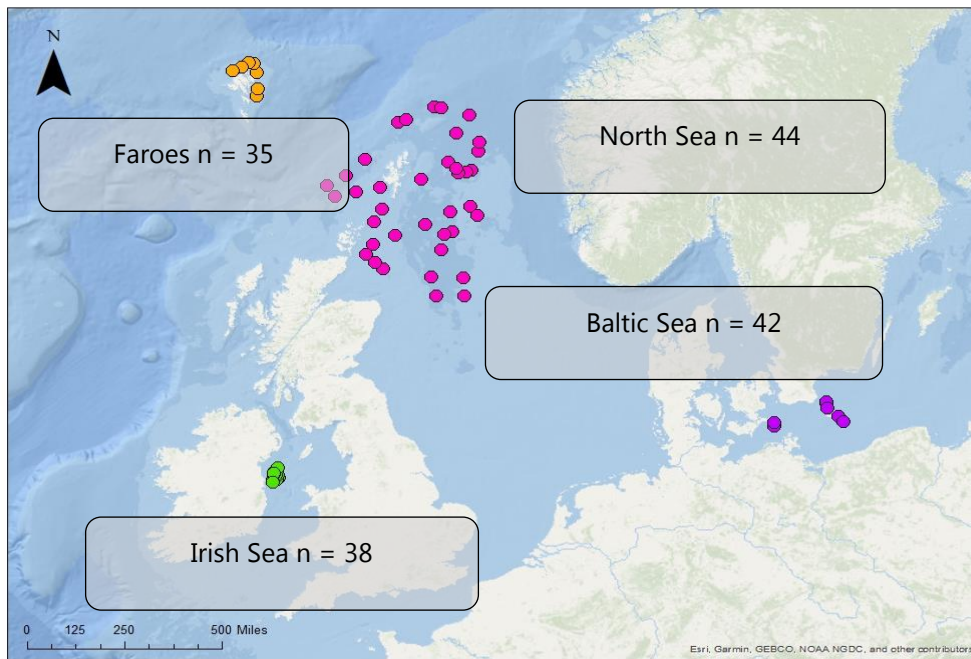


Figure 7 - Stable isotope compositions of *G. morhua* sampled from 4 fishery locations

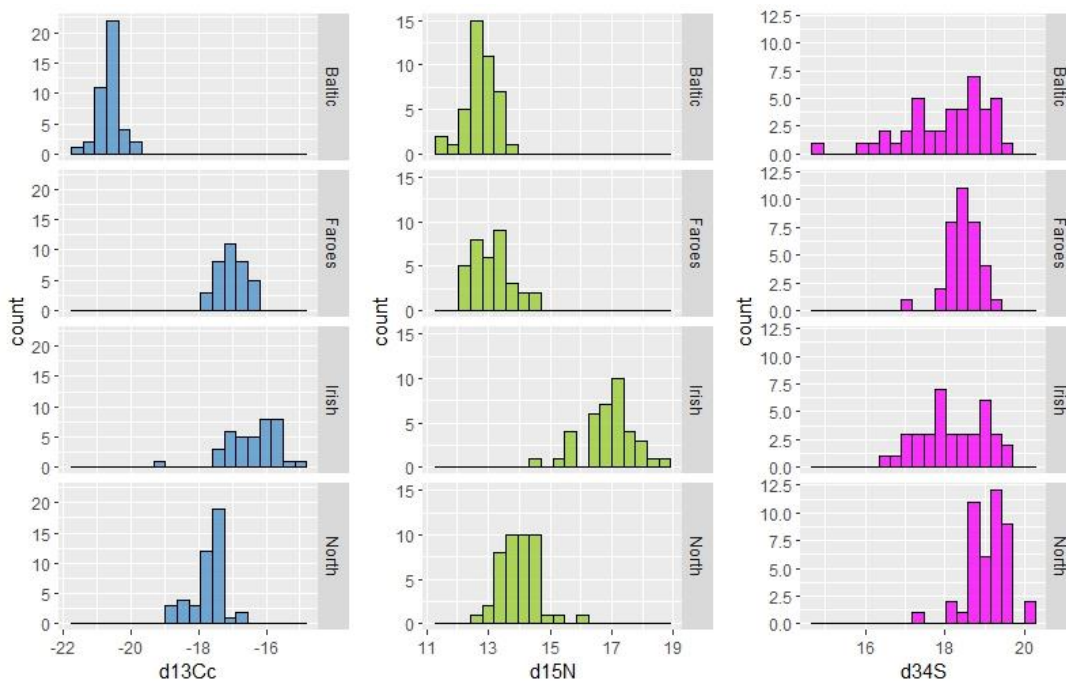
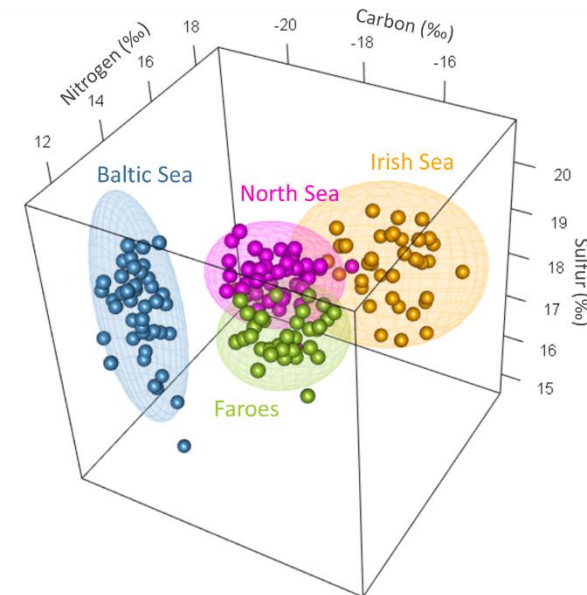


Figure 8 - 3D scatterplot showing stable isotope compositions of *G. morhua* sampled from 4 fishery locations



These data were used to calculate the assignment accuracy associated with assigning fish back to known origin based on a Monte Carlo resampling method. 25% of all samples were randomly extracted and held as test samples. Test samples were then assigned back to their most likely origin based on the isotopic distributions of the remaining 75% of the cod dataset, this re-sampling and classification steps was repeated 1,000 times.

The average accuracy of assignment of cod to known location for all four regions was 94.8% using all three stable isotopes (carbon, nitrogen and sulphur). Cod from the Baltic Sea were most accurately assigned, as would be expected given the isotopic distinctiveness of the Baltic Sea, with an average success rate of 96.9%. The lowest assignment accuracy was for the Faroes with 92.8% of cod correctly assigned, and most mis-assignments occurring to the adjacent North Sea. Using only carbon and nitrogen stable isotopes the average assignment accuracy for all locations was 95.1%, which is very similar to that with three isotopes. This suggests that sulphur is not necessary for high accuracy of assignment for these locations, but it could have more importance for other fishery areas in the north east Atlantic.

It is possible to achieve high classification success using a single reference dataset across broad ocean basin scales. Combining stable isotope with genetic methods is likely to result in higher levels of accuracy and precision than can be achieved using either method independently.

3.3 Regional seas-scale assignment: UK shelf case study

Assignment accuracy reduces with increased spatial precision demands, and also with increased separation between reference and test data. The relative difficulty of assessing geographic origin among sea areas within the UK shelf was demonstrated using uncontrolled samples by analysing data collected opportunistically and reported in Jennings and Cogan (2015). Jennings and Cogan

report a dataset of isotopic compositions of over 3,000 fish from 13 species captured from across UK shelf seas. To date this is the main reference dataset available to assess spatial variance in isotopic compositions across UK seas. Unfortunately, all reported samples from the North Sea were collected over a 5 year span from 2001 to 2006 whereas all other fishes were sampled from the Irish, Celtic Sea or English Channel in 2010. In addition, all North Sea samples were collected from within an unreported and restricted region in the north central North Sea.

Attempting to assign fishes back to known origin using linear discriminant analyses and ignoring isotopic variance associated with species, body size and date of collection results in relatively low overall accuracy of approximately 55% correct assignment among sea areas (Irish Sea, English Channel, Celtic Sea, Southern North Sea, and Northern North Sea). This value increased to c.60% when analyses are limited to fish caught in 2010. However, limiting the analyses to single species collected across the Irish, Celtic and English Channel in 2010 offers a more realistic sense of the degree of geographic discrimination possible.

Below a breakdown of classification accuracy is provided for all species where more than 20 individuals were available from at least 3 sea regions.

Plaice (*Pleuronectes platessa*):

The sample distribution is highly unbalanced, with samples dominated by those from English Channel. The overall assignment accuracy was 70%: Samples from the English Channel were assigned back to the English Channel with high accuracy (94%) and those from the Irish Sea were correctly assigned 72% of the time. Samples from the Celtic Sea and Southern North Sea, both tended to be grouped with English Channel origin fish. A balanced sample design is needed to reliably assess the ability to assign plaice to origin at spatial scales of ICES sea areas.

Dab (*Limanda limanda*):

Similar to plaice, although with a slightly more balanced sample distribution. High assignment accuracy to English Channel (94%) and moderate accuracy to the Irish Sea (67%) but Celtic Sea and Southern North Sea samples are again grouped with those from the English Channel.

Whiting (*Merlangius merlangus*):

Low accuracy overall, with c. 60% accuracy across all sea areas. This replicates impressions from other unpublished datasets suggesting that whiting is a challenging species for isotope-based traceability.

Gurnard (*Aspitrigla cuculus/Chelidonichthys lucerna/Eutrigla gurnardus*):

Mainly sampled from Celtic and Irish Sea with good accuracy distinguishing between those two regions: Samples were assigned with 86% and 77% accuracy respectively, most false assignments were to the Celtic Sea, intermediate between Irish Sea and English Channel.

Long rough dab (*Hippoglossoides platessoides*):

Sampled from only the Celtic and Irish Sea – accurate discrimination between these regions with 91 and 100% correct assignments respectively.

Anglerfish (*Lophius* species):

While the coverage was rather poor within the Jennings dataset, discrimination between Celtic and Irish Sea samples was reasonable with 88% and 75% correct assignments respectively.

Haddock (*Melanogrammus aeglefinus*):

Also good discrimination between Celtic and Irish Sea (84% and 80% correct assignments) but Channel-caught samples group with Celtic Sea samples.

Lemon sole (*Microstomus kitt*):

Relatively low accuracy across Celtic and Irish Seas and English Channel with only 60% accuracy overall and highest accuracy for English Channel fish at only 71%.

It should be stressed that the results outlined above are illustrative, based on opportunistic, and unbalanced sampling with only two isotope tracers. **At present a coherent dataset assembled explicitly to assess the accuracy and precision possible using stable-isotope methods is lacking.**

In summary, discrete assignment tests using stable isotope tracers offer similar levels of assignment accuracy to genetic tools (another technology considered to be advanced in this field), when applied on ocean basin scales as shown with cod fisheries. At higher spatial precision, such as within the UK shelf seas accuracy will be reduced, but at present we do not have a well-balanced, directed dataset to test the true accuracy and precision possible at UK shelf scales. A combined isotopic and genetic analyses will likely create highly accurate assignments, combining tracers based on population ancestry (genetics) and individual behaviour (isotopes).

A major requirement for bringing the method to readiness is analysis of commercial fish of interest from known locations (this should be repeated on c.5 year frequency to maintain a current dataset).

3.4 Continuous assignment case studies

A unique benefit of geographic assignment using stable isotopes is the ability to estimate origin across all possible areas (i.e. to build maps of probable origin for any single sample or populations of samples). This is termed continuous assignment, and may be appropriate where there are no reference datasets available covering all possible origin sites.

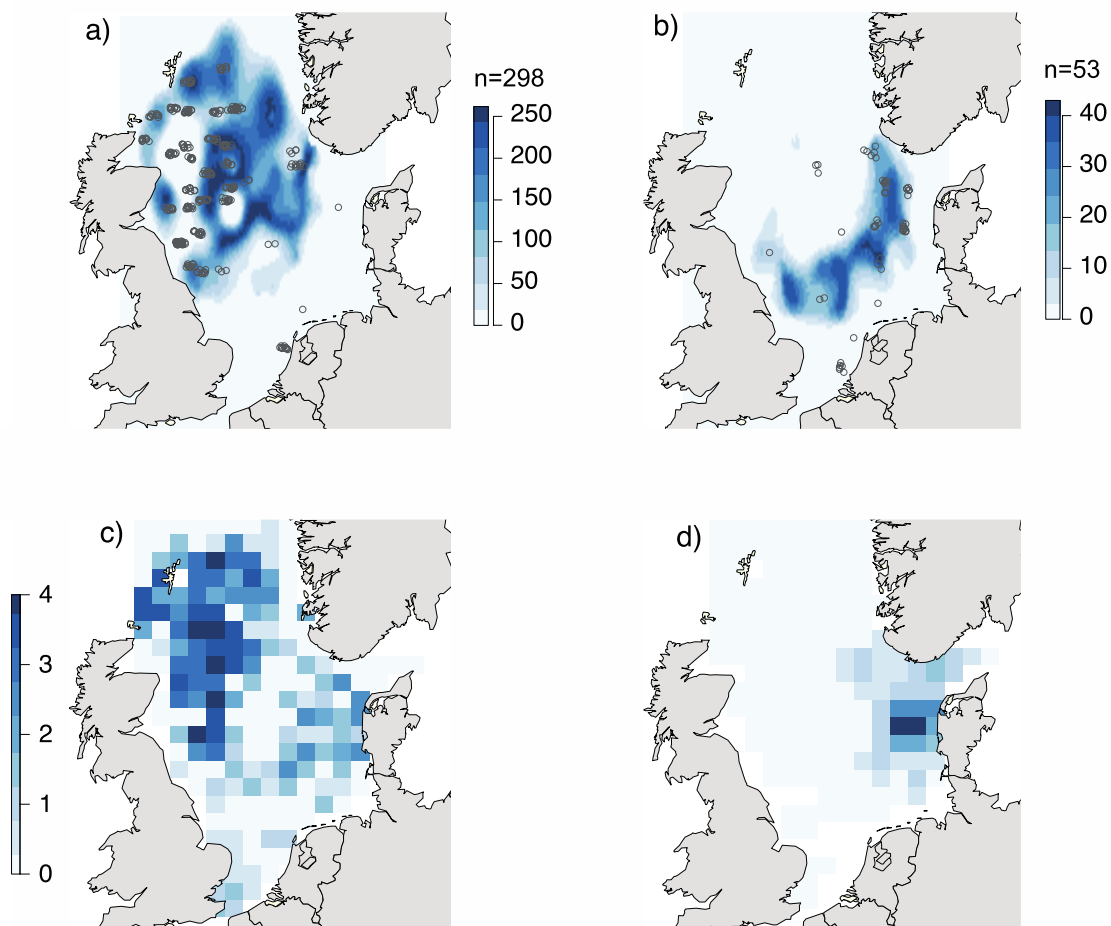
Continuous assignment draws on spatial models (isoscaples) as the reference against which test samples are compared. The outcome is a probability surface, which may be further reduced to a binary 'likely vs not likely' distinction based on pre-defined accuracy / precision thresholds (Trueman et al. 2017).

The concept of continuous assignments is illustrated using two datasets; herring (*Clupea harengus*) caught in known locations in the North Sea during ICES bottom trawl surveys, and scampi (*Nephrops norvegicus*) sampled from supermarkets in the UK in December 2018.

3.5 Herring

Atlantic herring were collected (n=351) at known locations across the North Sea in September 2011 as part of the International Bottom Trawl Survey. Muscle tissue carbon and nitrogen stable isotope values were determined and the fish were then assigned to their most likely origin based on our North Sea carbon and nitrogen isoscapes. The individuals were separated into two size/age classes, adults (n=298; Figure 9a) and juveniles (n=53; Figure 9b). Assignment surfaces of each individual were summed to display the population assignment area. Results were then compared against the spatial distribution of reported landings of adult herring (Figure 9c) and the estimated biomass of juvenile herring (Figure 9d) during the same year. Both adult and juvenile populations were able to be assigned back to their known catch locations, which correspond with the fisheries landing data and estimated biomass data (Trueman et al., 2017).

Figure 9 - Comparison of isotope-based feeding area assignments (a,b) and fisheries survey data (c,d) of adult and juvenile Atlantic herring (Trueman et al. 2017)



3.6 Scampi.

Nephrops norvegicus (n=85) purchased as breaded whole tail scampi products from UK supermarkets in January 2019 were sampled. All products were labelled as UK

or Scottish origin. The most likely catch locations using continuous surface assignment methods were estimated based on jellyfish isoscapes (Figure 10). Isoscape assignment indicated that of UK waters the central northern North Sea *nephrops* trawl fishery in the Fladen ground (Fishery Unit 7 in (Ungfors et al. 2013) – Figure 11) was a statistically more likely catch origin rather than the inshore West Scotland, Irish Sea or Clyde fisheries.

Figure 10 - Likely catch locations for 85 commercial (supermarket-bought) samples of processed scampi labelled as UK or Scottish origin. Colours and scale bar indicate the scaled probability of origin (in %) for each cell summed over all 85 sampled scampi.

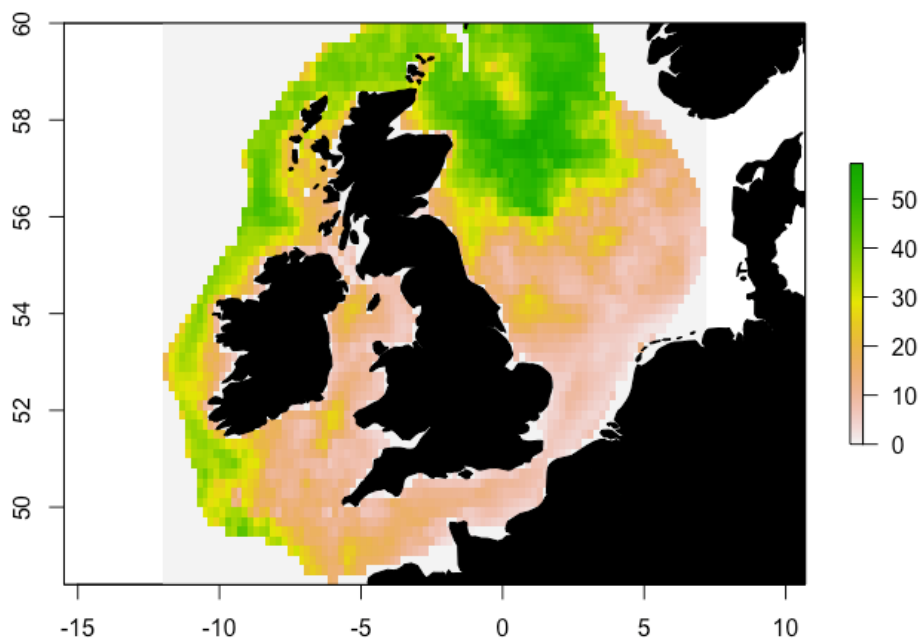
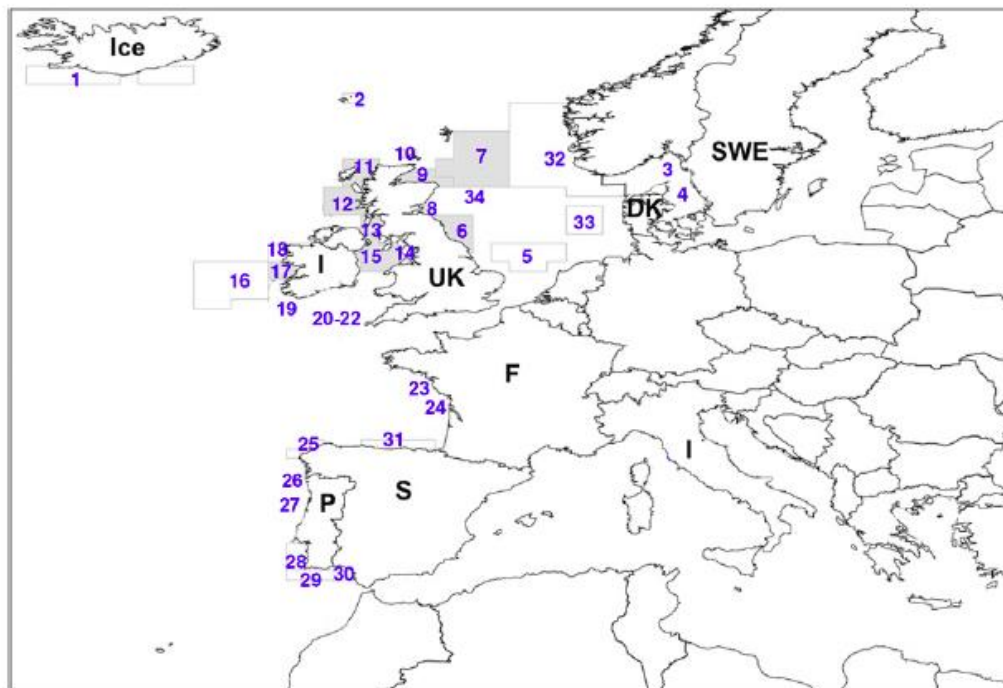


Figure 11 - Main Scampi fishing sites (Ungfors et al., 2013).



3.7 Commissioned tests: anglerfish and sole

Discrete and continuous assignment methods applied to anglerfish and sole samples conducted in April 2019:

75 samples of anglerfish and 4 sole were provided and analysed to determine $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values with two aims:

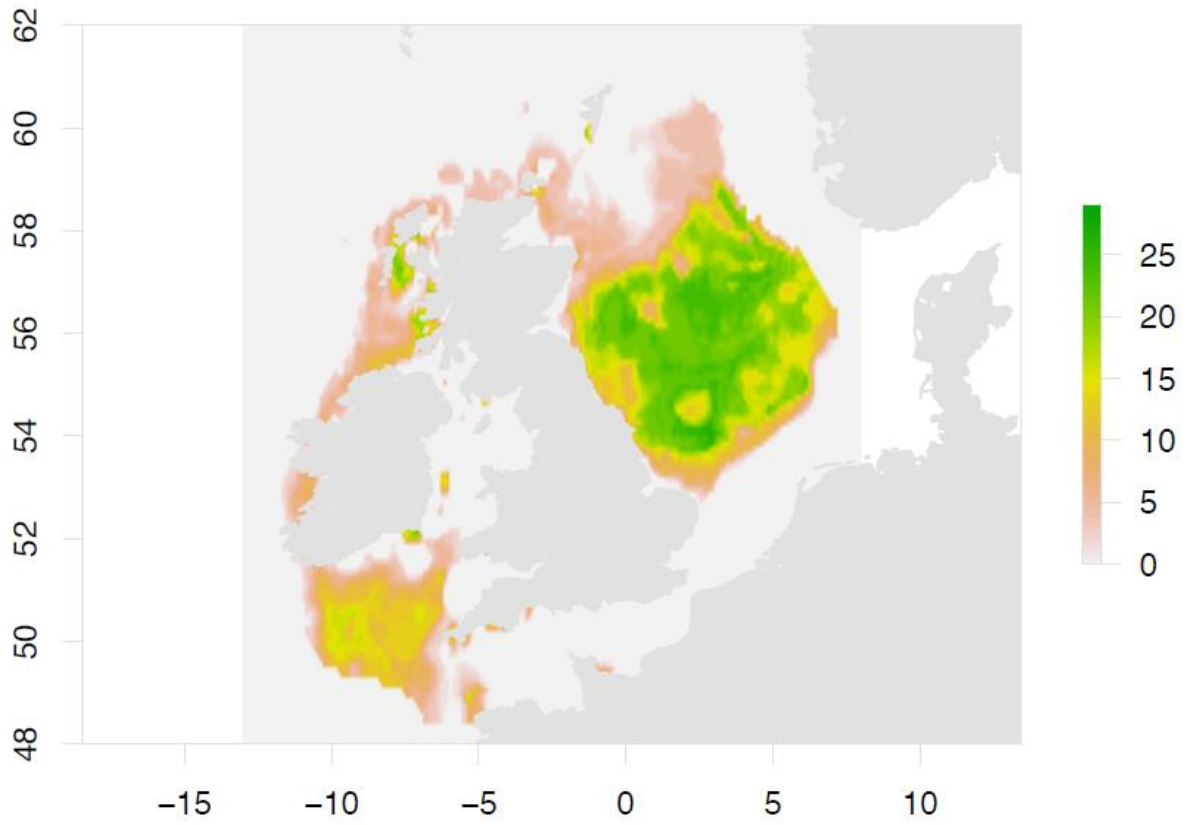
- (1) can samples from different known origins be discriminated using stable isotope data?
- (2) can the known location be inferred based on existing reference datasets?

All provided samples were analysed for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ isotope values (raw data attached as Annex 1 and 2).

Anglerfish from ICES VIIIE were provided across a wide range of body sizes (from 11cm juveniles to 125cm adults) which introduces additional variance in isotopic data, but with no correction for body size the populations of samples recovered from VIa and VIIe could be discriminated from one another with 72% accuracy. Attempts were also made to assign anglerfish data to the continuous isoscape with best predicted estimates for calibration offsets between jellyfish and anglerfish (Figures 12 and 13)

Figure 12 - Inferred spatial origin across the whole UK shelf sea for anglerfish caught in ICES VIa using CN (A) and CNS (B) isoscape models

a)



b)

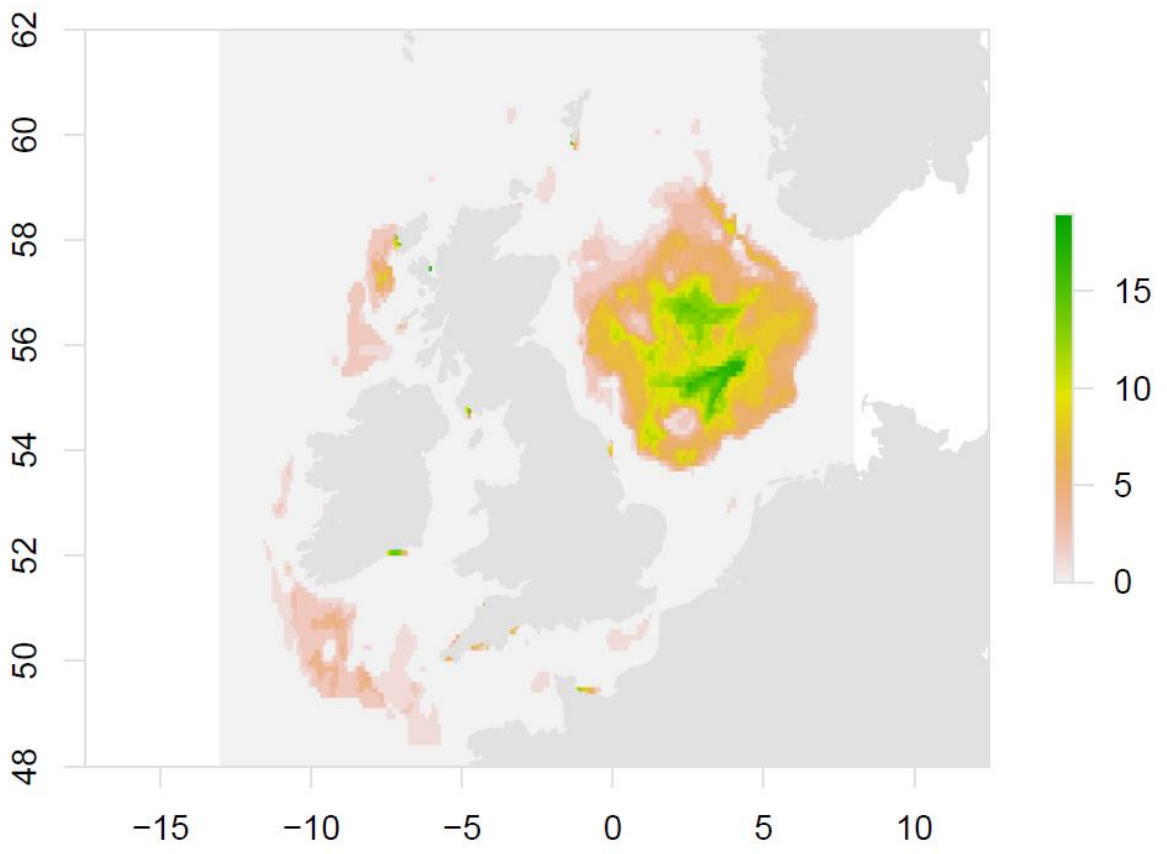
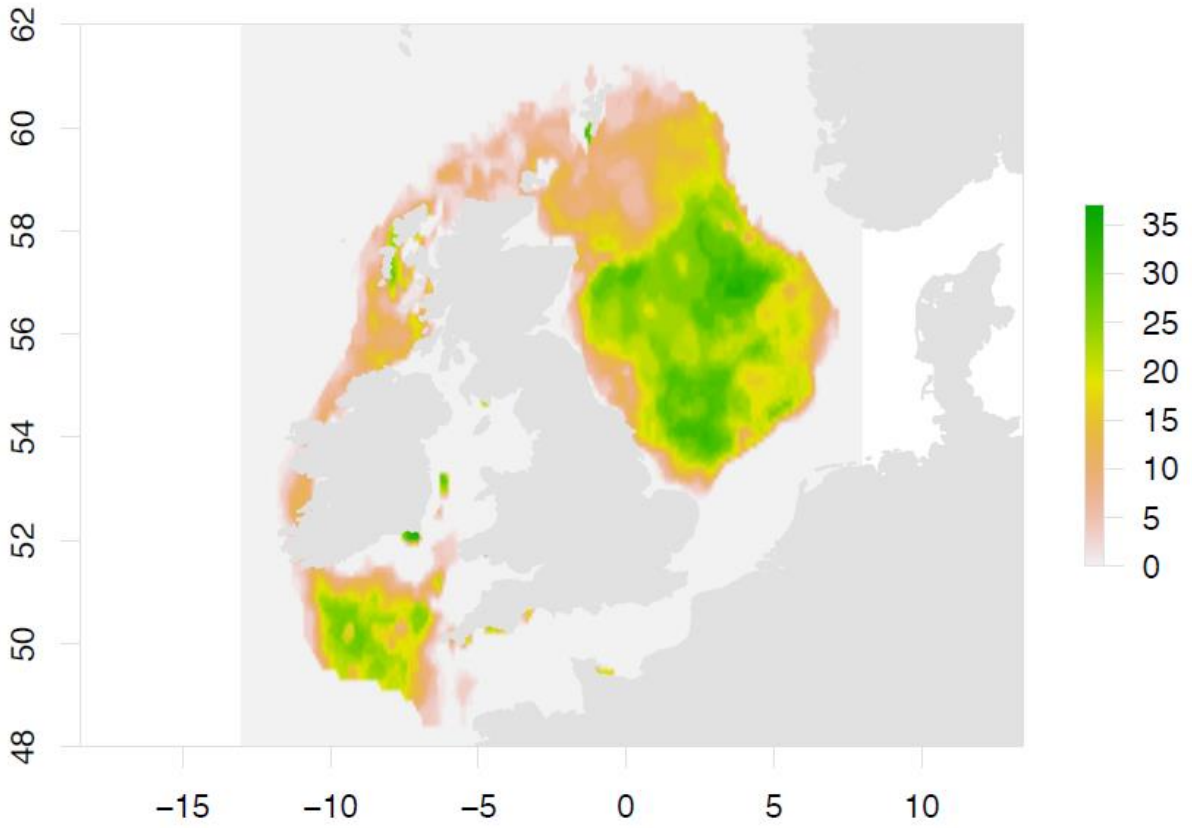
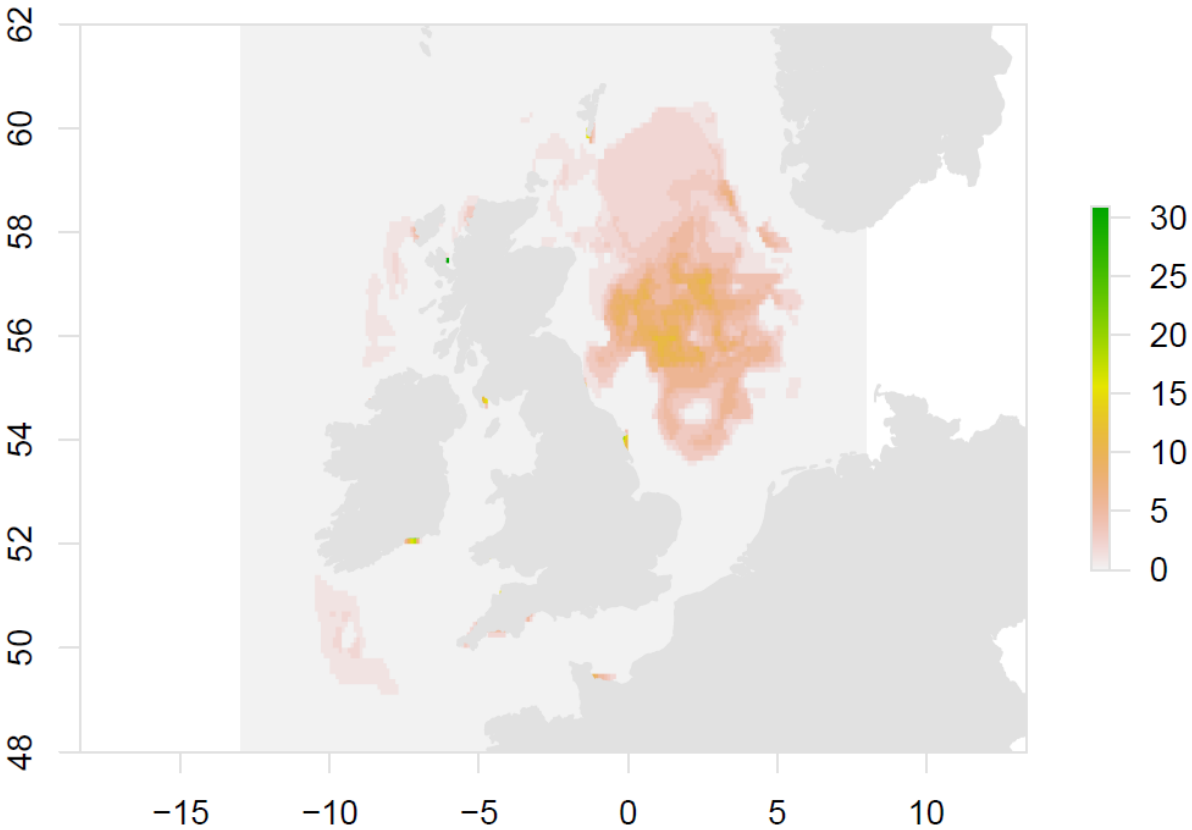


Figure 13 - Inferred spatial origin across the whole UK shelf sea for anglerfish caught in ICES VIle using CN (A) and CNS (B) isoscape models

a)



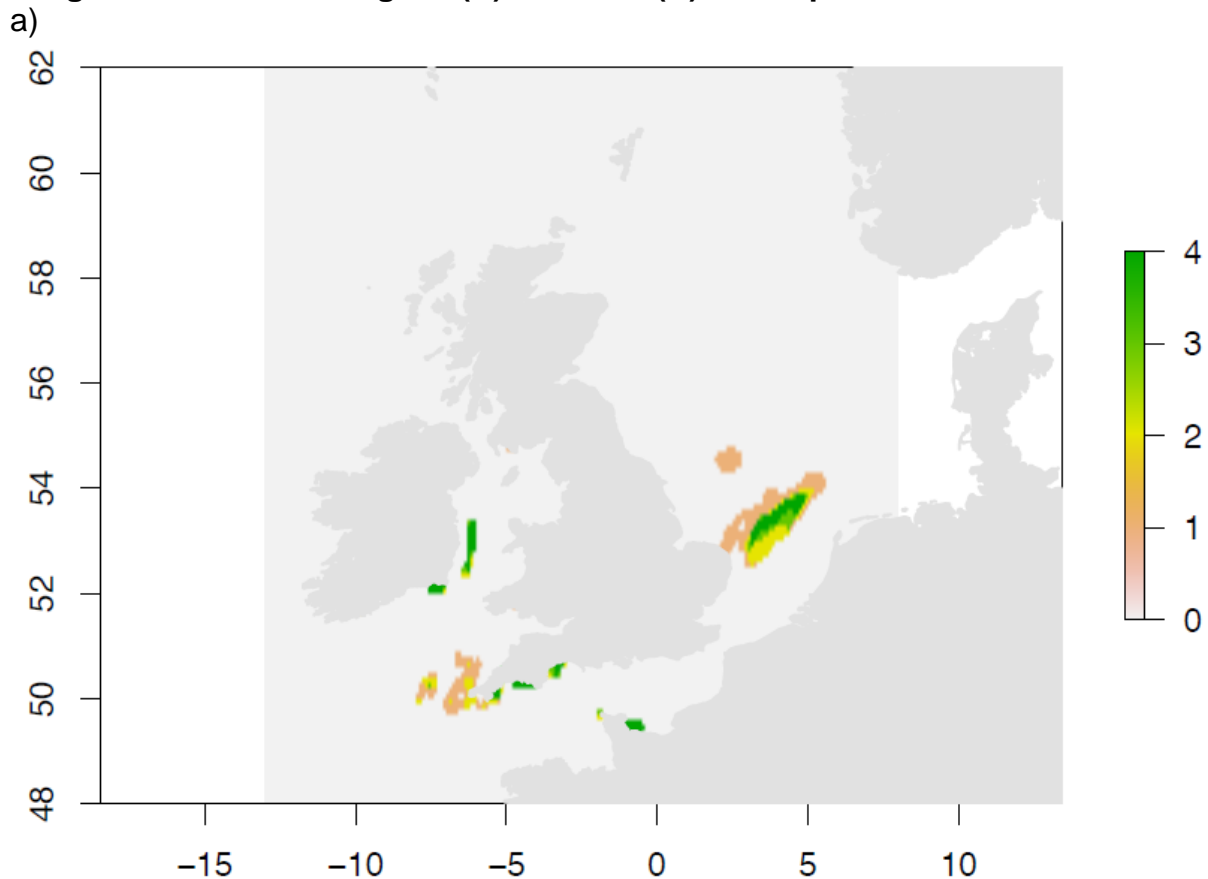
b)

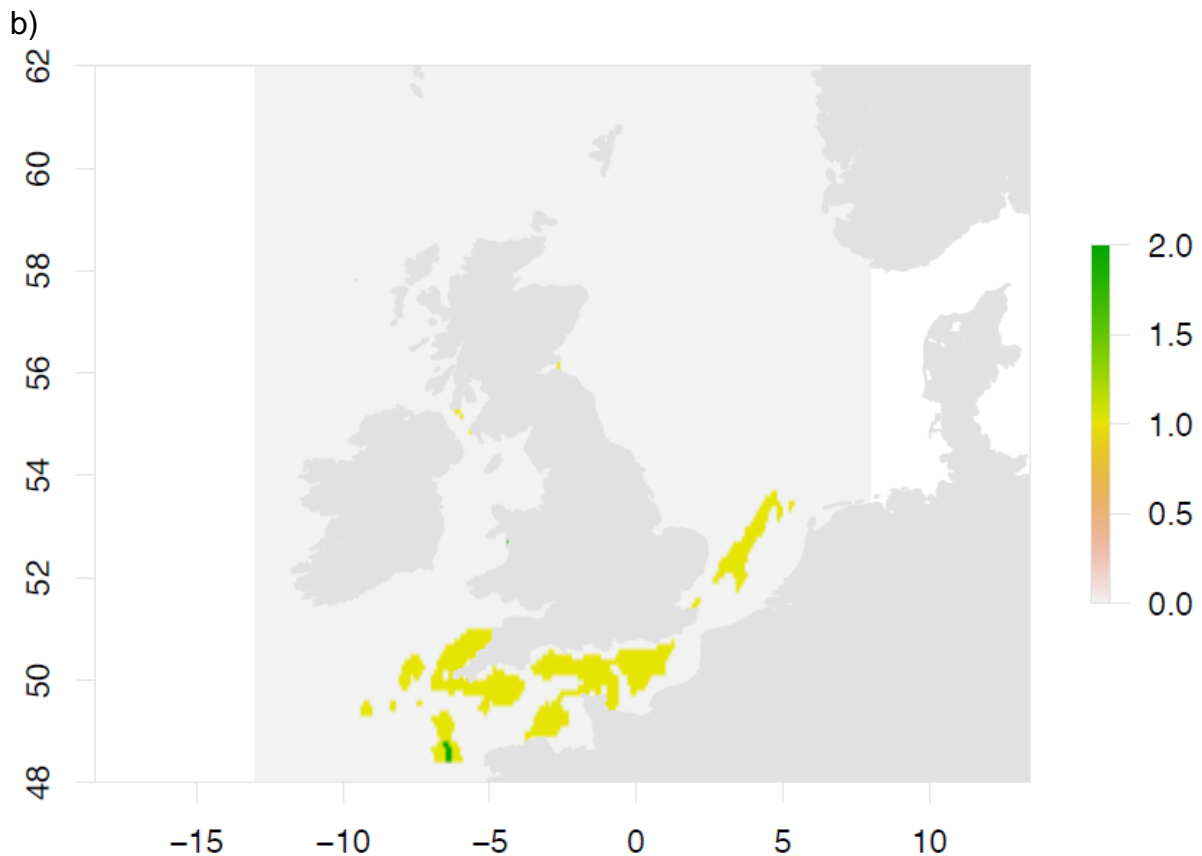


For fish caught from ICES VIa, the true catch location is included in the identified regions of highest probability of origin but a large region of the central North Sea has similar predicted isotopic compositions (Figure 12). Consistent with the relatively low levels of isotopic separation between the two populations (only 70% accurate discrimination) Samples from ICES area VIIe show similar potential regions of origin to anglerfish sampled from VIa, with a larger potential origin area within from the Celtic Sea (VIIe-j) (Figure 13). The assignment precision could be improved with better calibrations between anglerfish and jellyfish. Alternatively, these data could be used to establish a calibration which could be subsequently applied to infer origins.

Only 4 samples of sole recovered from ICES VIIe were provided so assignment accuracy could not be assessed. Instead we attempted to identify most likely catch locations based on a best estimate of isotopic offset between reference jellyfish isoscapes and sole (Figure 14).

Figure 14 - Inferred spatial origin across the whole UK shelf sea for sole caught in ICES VIIe using CN (A) and CNS (B) isoscape models





Best estimate assignments reproduce ICES VIIe as most likely geographic origins. Again the known catch ICES region is contained within the indicated likely origin areas. But more data are needed to establish appropriate calibration offsets between jellyfish-based isoscapes and sole muscle.

4. Future work

The technological readiness level for stable isotope methods will be best advanced through the development and maintenance of reference datasets particularly assessing species-specific isotopic variation across key locations.

The work conducted to date has demonstrated that stable-isotope based methods have the potential to provide traceability and spatial-origin information at practically useful levels of accuracy and precision. However, there is not currently sufficient datasets of the isotopic compositions of known-origin fish of commercially relevant species. This is critical to enable one to:

- (1) determine the true accuracy and precision associated with discrete assignment approaches while considering realistic distributions of isotopic compositions within regions
- (2) identify the spatial scale and regional contrasts most effectively discriminated using stable isotope approaches (i.e. confirming testing predictions as illustrated in Figure 5)
- (3) establish species-level calibrations to isoscape models and employ continuous surface assignment methods.

Assembling required datasets is relatively simple though collaboration with ICES international fisheries surveys.

As an example, deriving species level calibrations across 11 ICES fishing areas abutting the UK coast for 10 commercially relevant species could be achieved with sample sizes of up to 100 individuals per species per ICES area (depending on the size of the area). Given that all species are unlikely to occur in all ICES areas, a rough estimate of 6,000-7,500 isotope measurements would provide a robust and comprehensive reference dataset that could be used directly to assess origin of the target species, or to refine estimates of likely isotopic distributions for ecologically similar species not included in the initial reference dataset. Likely costs for isotope analyses depend on laboratories used and involvement of academic partners, but may vary from <£10 to >£20 per sample for combined CNS analyses and potentially c.£5 per sample for just C and N isotope analyses. The analytical costs associated with bringing isotope –based traceability to full operational readiness levels are therefore likely to be on the order of £50 - £75K not including staff time. Smaller scale projects could be developed to target fewer species or restricted areas. Critically such sampling programs could (and should) be progressed in association with other methodologies, especially genetic approaches, with parallel development of a statistical assignment framework incorporating multiple independent lines of evidence.

5. Summary

Stable isotope based approaches are firmly established as effective and reliable methods to trace spatial origin in terrestrial forensics (widely used in food and agriculture, explosives, drugs trade, homicides etc).

A major advantage of isotope based methods is the predictability of spatial gradients in isotopic ratios. This means that isotopic differences between or among regions can be explained mechanistically and linked to environmental context. Continuous spatial statistical models can also be built to map isotopic gradients across space (isoscapes).

In marine systems isotope-based traceability is less commonly used, probably because of the relative practical difficulty of establishing known-origin reference datasets.

Recent developments in the generation of marine isoscapes and increased interests in geographic traceability of marine animals have led to rapid increases in the technological readiness of stable isotope based traceability methods in marine systems, and some commercial traceability companies offer isotope-based geographic profiling services.

In marine systems isotopic variability is mainly expressed through biological processes associated with fixation of nutrients by primary producers and subsequent transfer of spatially-varying isotopic compositions through food chains.

Biological processes linked to trophic level, diet, isotopic fractionation and movement behaviour complicate the interpretation of stable isotope values in a

spatial context. Some expert knowledge is needed to identify when combinations of species and regions provide good candidates for isotope-based traceability approaches.

The UK shelf seas are most well-studied marine regions globally in terms of spatial variation in stable isotope compositions, and strong, systematic and temporally stable isotopic gradients exist across UK shelf seas.

The methodology and mechanistic understanding of isotopic variability is in place but we currently lack reference datasets of known-origin fishes needed to establish the maximum accuracy and precision available using stable isotope-based methods for traceability in a UK context.

The costs associated with bringing isotope-based methods to full operational readiness are relatively low, building on strong existing datasets and expertise.

All indirect traceability approaches are best used in conjunction. Stable isotope and genetic approaches are particularly well suited for combined analyses as both yield independent spatial inferences, isotopes based on the location of the diet of the individual, genetic inferences based on the historical location of the reproductive population.

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