Marine
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Organisation

Evaluating and advancing methodologies to validate the origin of capture of fish: Trace Element Technologies (MMO1194)

MMO1194: Evaluating and advancing methodologies to validate the origin of capture of fish: Trace Element technologies
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Executive Summary

Using the trace element composition of animal tissues as a natural tracer for spatial origin depends on the assumption that consistent spatial variations in the relative and absolute composition of trace elements exist in the natural environment, and that these spatial variations in elemental compositions are passed into animal tissues with relatively little or predictable biological alteration. If these assumptions are met, then the elemental composition of animal tissues from an unknown or uncertain location can be compared statistically to reference databases of elemental compositions in tissues or environmental matrices from discrete known locations.

The use of trace elements as natural tracers for spatial origin is therefore limited by three main factors:

  (1) the nature of spatial variation in trace elements in the natural environment
  (2) the degree of biological transformation of relative and absolute elemental concentrations between the environment and the tissue
  (3) the availability of suitable reference datasets

While trace element compositions of fish tissues have been routinely reported from UK waters (especially in relation to pollution and environmental toxicology), there are currently no substantial, consistent datasets available to predict spatial variations in elemental concentrations at a local or regional level.

The technological readiness level for trace element methods will be best advanced through a combination of predictive spatial modelling and the development and maintenance of reference datasets. Integration of trace element and other natural tracers is likely to improve the potential for traceability 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 is concerns trace elements, one of four technique classes previously identified as promising by the MMO along with stable isotopes, trace elements and genetics.

This report largely synthesises existing knowledge regarding spatial distribution, biological control and study design protocols associated with trace element based traceability of fish products, their impacts on the practicalities of using trace elements 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 elemental variability and biological transformations in general.

- Section 3 describes study designs associated with use of trace element tracers for geographic origin, their database and analytical requirements, with specific focus on applications for marine foodstuff traceability.

- Section 4 provides a personal view on research needed to enhance the technological readiness level of the trace elements method as tracers of geographic origin in the UK marine foodchain.

The main (and big) advantage of using trace elements as geographic tracers is the number and variety of elements potentially available. Analysing a wide range of elements with differing geochemical behaviour increases the number of dimensions across which the chemical compositions of animal tissues can vary depending on location, and maximises the potential for geographic variability in concentrations (i.e. increases the spatial signal). However, the potential for variation additional to the regional pattern under study, such as local spatial-temporal variations or physiological variability is also increased – thereby increasing noise in the context of geographic traceability.

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 complexity influencing the abundance, distribution and bioavailability of trace elements in the natural environment limits the ability to predict variations in elemental concentrations *a priori*. Consequently, trace element-based traceability studies are based on comparisons between the test samples and databases of tissue compositions from known discrete reference sets. This means in practice that reference databases must be assembled for every study in question, and that these databases must either be demonstrated to be temporally stable, temporally specific, or must be updated regularly. **The practical and financial implications of building and maintaining suitable reference databases is the largest barrier to the effective use of trace element based geolocation.**

The level of implementation of the broad concept of trace element was analysed 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.

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 laboratory
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)

Overall trace element methods have shown value in experimental proof of concept analyses and laboratory conditions, and a wide range of case study examples have demonstrated mixed levels of success. The academic literature is assumed biased towards examples where trace element methods have 'worked'. In a UK context we lack a comprehensive assessment of the applicability and accuracy of trace element methods in the natural environment. Overall TRL therefore lies between 3 and 5.

2. Biogeochemical context[1]

2.1 Trace Elements

Trace elements are loosely-defined as elements present in tissues at concentrations under (100ppm or 1mg/kg), although this concentration is often exceeded in certain organisms and tissues and under different environmental settings. Using the loose definition above, there are more than 50 elements that can be detected in animal tissues using modern mass spectrometry methods and therefore potentially available as geographic tracers. In practice, however, the suite of elements potentially suitable as geographic tracers is limited to elements whose concentrations in animal tissues are both readily detectable and not under strict physiological control.

Animals may be considered as net accumulators of a trace element if the net uptake of that element exceeds the net excretion. Many biologically essential elements are not free to vary in relation to environmental gradients, and these elements are not generally strongly accumulated in tissues. Essential elements include sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and, to a lesser extent, copper (Cu), manganese (Mn) and zinc (Zn). Essential trace elements are typically avoided in traceability studies either because of their limited variation among sites due to their concentrations in tissues being regulated within strict physiological limits, or because of the potential for physiological (rather than spatial)-related variance. Fish are generally considered to regulate the tissue concentrations of many trace metals within strict tolerances, reducing the potential for variation related to geography ('signal') compared to physiological 'noise' (Sturrock et al., 2015).

The value of trace-element based tools for geographic traceability is enhanced where animal tissues are strong net accumulators in proportion to the concentration of elements in the local environment. Non-essential elements, an element whose tissue concentrations are under more limited physiological control, form the core suite of elements used as geographic tracers. Fish have less ability to regulate concentrations of non-essential (frequently toxic) elements such as cadmium (Cd) arsenic (As), lead (Pb) and mercury (Hg). Consequently fish tissues may act as net proportional accumulators of these elements, providing a signal for traceability. The spatial distribution of bioavailable Cd, As, Pb and Hg in UK waters is closely linked to human activities, especially mining and mining legacy, industrial and sewage waste disposal and dredging (Rainbow 2018). To fully appreciate the potential and complexity of using trace elements as geochemical markers, it is useful to briefly consider their aquatic and biological chemistry.

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[1] Rainbow (2018) provides a thorough and comprehensive review of the biochemistry of trace elements in the natural environment and living organisms, within a UK context. Rainbow's text summarises much of the essential biochemical background and identifies key historic datasets available for giving context to trace element based analyses in a UK context. Unsurprisingly, marine habitats are significantly less well characterised than estuarine, freshwater or terrestrial habitats.

2.2 Aquatic and biological chemistry

All elements can be broken down into geochemical groupings. In the 1960s, chemists developed a concept known as "hard and soft acid and base" (HSAB) theory. HSAB theory is useful for predicting the affinities and behaviours of elements in complex systems. 'Hard' acids and bases are typically small ions or molecules with relatively high charge density that are weakly polarisable. 'Soft' acids and bases tend to be large and strongly polarisable. The value of this concept is that acids and bases form strong bonds when binding with similar counterparts (i.e. hard acids form strong bonds with hard bases and soft acids with soft bases). In complex and dynamic media like seawater and blood plasma, the number of available ligands outweighs the number of metal ions. In these cases, strong bonds will tend to dominate the speciation of any metal ion, and the behaviour of metal ions can be predicted (Williams, 1971).

The marine system is a dynamic environment with elements constantly added, removed and recycled through biological, physical and chemical processes (Hunter and Boyd, 1999). Yet global averages for element concentrations in seawater have remained remarkably conserved, particularly when compared with estuarine and freshwater systems. Many marine fishes, particularly open ocean pelagic species, therefore experience a relatively uniform physicochemical environment with limited potential for spatial discrimination using inorganic chemical proxies (Proctor et al., 1995), although there are some notable exceptions (e.g. Ashford et al., 2008). Coastal areas generally offer greater chemical heterogeneity due to upwelling, fluvial and anthropogenic inputs. The 'pollutants' that would typically contribute to geographic variation in costal settings (e.g. nickel (Ni), zinc (Zn), As, Pb, Cd, Hg) are, however, soft acid metals that are discriminated against physiologically and are often present in concentrations close to or below detection levels in many fish tissues (Hanson and Zdanowicz, 1999).

In seawater, hard metal acids such as Li^+ (Lithium), Mg^{2+}, Ca^{2+} and Sr^{2+} (Strontium) form strong bonds with $^{\cdot}OH$ radicals and are mainly found as hydrated free ions. These ions typically have a residence time longer than the mixing time of the ocean and consequently their concentrations tend to vary largely with salinity. If test sites differ strongly in terms of salinity, hard metal acids frequently provide effective tracers. By contrast, soft metal acids such as Cu^{2+}, Zn^{2+} and (to a lesser extent) Mn^{2+}, bind to softer bases in seawater such as Cl^- (Chlorine) and CO_3^{2-} (Carbonate Ion). These bonds are relatively weak, making soft metals reactive and readily removed from seawater (Henderson, 1984), so that regional variations in their concentrations can be established.

Figure 1: relative concentrations of selected elements in global average seawater compared to global average river water (data from Henderson 1984)
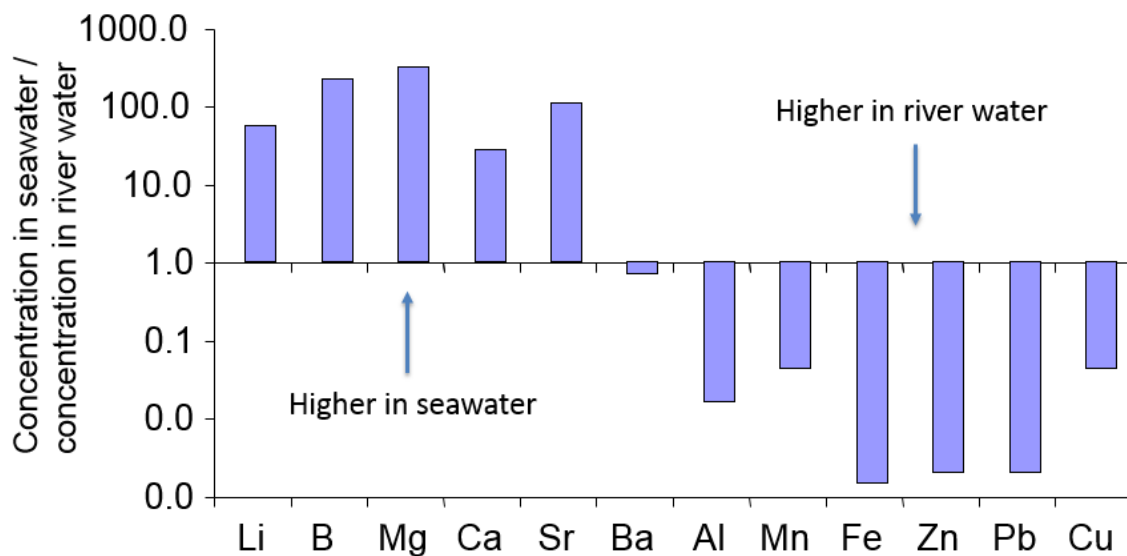
2.3 UK Waters

In a UK context, Rainbow (2018) provides an extensive introduction to the existing geochemical literature describing spatial and temporal variability in trace element compositions in sediments, water and biota in UK environments. Note that many trace element analyses have been conducted in a historical context of relatively intense chemical pollution, and may not reflect modern conditions. In coastal settings, at a course scale, the potential for geochemical distinction between regions can be initially predicted based on (a) the geological context of the adjacent drainage basin and hydrology and (b) human inputs to the coastal environment. Broadly speaking, coastal regions draining limestone or chalk regions may be expected to provide geochemically contrasting coastal waters from regions draining sandstone or igneous bedrocks. Regions with long industrial heritages especially those draining mining regions or with heavy industry in coastal zones may provide distinct geochemical tracers especially in toxic metals such as As, Pb, Hg and Cd. While greater environmental heterogeneity in coastal areas generally increases the potential for discriminatory power associated with tissue trace element compositions, it is, unfortunately, usually at the expense of temporal stability (Elsdon et al., 2008; Gillanders, 2002; Walther and Thorrold, 2009; Rainbow 2018). For this reason, reference collections of tissue for relevant year classes and locations are recommended.

In an ideal world, spatial variation in natural chemical tracers would be strictly hierarchical – that is, local scale variability would be nested within larger spatial scale tracers (figure 2). Unfortunately this is rarely observed with trace element tracers, as major drivers behind variation, such as salinity act on local rather than regional scales. Combinations of trace element and stable isotope or genetic tracers may provide more hierarchical information, drawing on the advantages and strengths of each approach.

Figure 2: A cartoon summary of hypothetical ideal hierarchical structure of spatial variation in natural tracers

Hierarchical variation in otolith chemistry -

1000's of km

Variation at small spatial scales is less than variation at higher spatial scales

100's km

Hierarchical variation is rarely observed

10's km
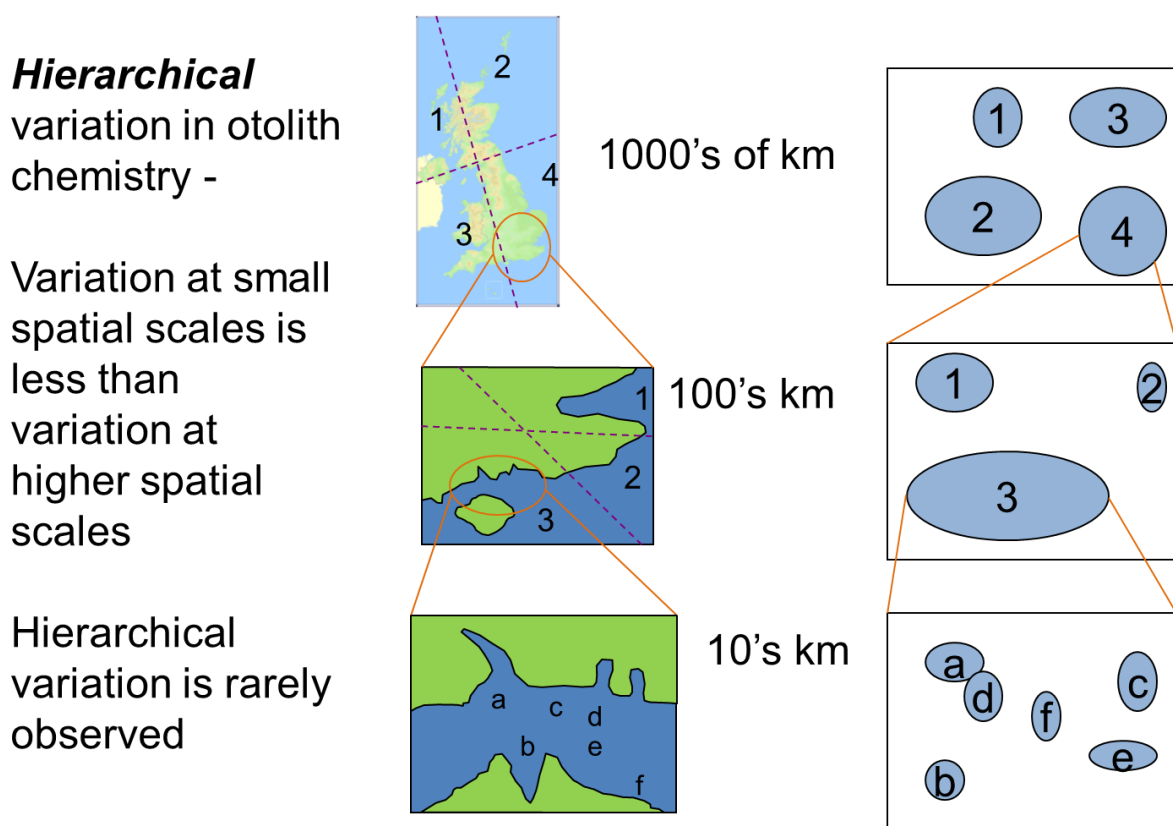
Trace element tracers tend to vary at small, local spatial scales - hierarchical structure may be possible with coupled tracers (e.g. stable isotopes, genetics + trace elements).

The relative concentrations of trace elements dissolved in local waters do not necessarily reflect the potential for regional differences in tissue chemistry because of physiological influences on trace element uptake and distribution among tissues

2.4 Physiological influences

The absolute and relative concentrations of trace elements in animal tissues are physiologically regulated. The absolute and relative concentrations of trace elements in animal tissues therefore reflect both differences in the external environment, and any physiological effects on element uptake and excretion. Our knowledge of physiological influences on tissue metal concentrations in non-human animals is generally rather limited. In a marine fisheries context, the trace element compositions of fish otoliths (calcium carbonate structures in the fish ear) have long been used as geographic markers. Otoliths are especially attractive tissues for trace element-based spatial studies as the organic-mineral composite structure hosts a wide variety of trace elements (Sturrock et al., 2012). Most of what is known about trace elements

as geographic tracers in marine animals comes from the otolith and to a lesser extent, environmental toxicology literature.

Relatively little is known of the bioavailability of soft and hard metal acids to uptake in marine fish, but it is likely that soft and hard metal acids will be fractionated from one another during transport across the gut (See Rainbow 2018 for a modern review of bioavailability of trace metals). Hard metal acids are largely transported as free ions in plasma or weakly bound to small molecular complexes, blood albumins or globulins. Calcium (and Ca homologues) may also be strongly bound to carboxyl residues in Ca-binding proteins, but more than 40% of all Ca is estimated to be present in plasma as the free ion (Andreasen, 1985; Hanssen et al., 1989). By contrast, soft metals such as Cd, As, Pb, Cu, Zn and possibly Mn are actively bound to histidine, methionine and cysteine residues in plasma proteins and used in metabolic reactions or actively transported to the liver where they are excreted (Watanabe et al., 1997, Rainbow 2018). Less than 10% of total blood Cu and Zn are estimated to be present as free ions (Williams, 1971). Therefore hard and soft metal acids ions are very likely to be fractionated from one another within the blood plasma. This is important as any process that fractionates metal ions from one another will disrupt the relative concentrations of elements so that the relative tissue concentrations in tissues will not reflect the relative concentrations in water.

The speciation (the chemical form) of metal ions within blood plasma is likely to be dynamic, and at any time a proportion of all metals will be held as free ions. It is likely that blood chemistry, particularly the composition and relative abundances of blood proteins, will influence this proportion and thus affect the distribution of metals within tissues (Sturrock et al., 2012, 2015, Rainbow 2018). Physiological variation in the composition of blood plasma proteins is more likely to influence the soft metal acids as their ions are more strongly associated with blood proteins. Different populations or samples of fishes, potentially with differing physiological status, life stage or genetic make-up may fractionate elements differently, so that the relative difference in elemental fingerprints between locations may also not reflect the relative difference in bioavailable water compositions. However, while this is a theoretical concern, there is little data available to quantify the extent to which population-scale variations in elemental fractionation are sufficient to outweigh geographic variations.

Another factor potentially complicating the interpretation of tissue trace element compositions is that the residence times of individual elements within the fish are likely to vary. This is particularly important in mobile fishes where movements across environmental gradients may occur over timescales shorter than the residence time of trace metals in tissues, further complicating the relationship between tissue chemistry and that of the surrounding environment.

Elements that are constituent parts of functioning enzymes or structural tissues are likely to have relatively long mean residence times in the body due to the continued recycling of enzymes and tissues. By comparison, elements with no major structural or physiological role may be likely to be removed relatively rapidly, resulting in low residence times. The quintessentially toxic (accumulating) metals such as Cd, Pb, As and Hg may have very long residence times as excretion mechanisms are relatively ineffective, and tissue concentrations may more closely reflect lifetime exposures.

The most detailed understanding of trace element uptake in fish tissues relates either to Pb, Hg, Cd, As and their relationship to anthropogenic pollution and human heath, or to studies of otolith chemistry. Relatively comprehensive experimental studies and reviews into the current state of understanding of the physiological influences and controls on trace element incorporation into fish tissues are provided by Campana, 1999, Sturrock et al., 2015, Elsdon, 2008 and Tzadick 2017. However, despite decades of scientific investigation, the precise mechanisms governing elemental incorporation into the otolith are still not fully understood. In a food chain traceability context, otoliths will frequently not be available (heads not attached), and there is much less understanding of spatial influences on the trace element compositions of muscle tissue. The implications of tissue selection will be discussed in section 3.

Metabolically active tissues such as muscle and liver are likely to respond more rapidly to changes in ambient water compositions than structural tissues such as otoliths or the skeleton

In a UK context, Rainbow (2018) provides an extremely thorough summary of the state of knowledge to date regarding the distribution and concentration of trace elements from UK estuarine and coastal waters. The available data is strongly focussed on pollutant metals Cd, Cu, Zn, Pb, As and Hg, reflecting both environmental and regulatory concern over anthropogenic inputs to the environment, and the greater potential for biomonitoring using elements that accumulate strongly in animal tissue. Consequently, a large proportion of the available data relating to fish (and invertebrate) metal concentrations relates to industrially contaminated sites such as Restronguet Creek in the Fal estuary, estuaries of the Severn, the Thames and the Clyde, Liverpool Bay and Poole Bay, and particularly between the 1970s and late 1990s. In recent years, reductions in discharges of metal-containing industrial waste and sewage have resulted in dramatic declines in pollutant trace elements in a wide variety of organisms, so that historic datasets are not valid predictors of current spatial variations in trace element compositions, particularly for those elements most likely to accumulate in fish tissues in proportion to environmental bioavailable concentrations.

Most existing data on metal concentrations in fish tissues from UK coastal and marine waters relates to historic anthropogenic pollution, and is not well suited for prediction of spatial variation in trace element concentrations across modern UK waters.

2.5 Summary on constraints

A priori prediction is extremely difficult and the likelihood of locations bearing a temporally-stable, 'unique' elemental fingerprint is unlikely given the wide range of factors influencing trace element compositions and the combinations of geographic and biochemical factors influencing bioavailable trace element concentrations. Consequently, trace element-based geographic provenance applications are most effective in the context of comparing an unknown sample to a reference dataset explicitly assembled for the purpose of geographic assignment. This is discussed more fully in the next section.

3. Study design considerations

3.1 Overview

As detailed in section 2, the complex nature of elemental variability in the natural environment, in particular the varied spatial scale of trace element heterogeneity, coupled with potential for physiological variations mean that *a priori* prediction of elemental compositions of fish tissues expected from different locations is extremely difficult. Consequently, trace element -based traceability studies are best suited to comparisons between tissue chemistry and reference samples drawn from known locations (Figures 3-5).

Because no single trace element composition is likely to have a unique geographic origin, one cannot assign a sample to a location confidently. However, if a test sample is chemically distinct from that in a suitable reference population, there may be good grounds for supposing it was not drawn from the same region, providing that the reference sample is suitably matched in terms of spatial and temporal variance within the test location, and potential physiological influences on uptake. The exact conceptual test performed is therefore whether measured trace element compositions are *inconsistent* with reference compositions or to assess which of a pre—defined set of possible locations presents reference trace element compositions most consistent with the test data.

3.2 Study requirements

The difficulty of obtaining adequately defined reference datasets is the single largest impediment to using trace element fingerprints for routine spatial traceability studies.

Trace element concentrations and bioavailablilties are relatively homogenous in open seawater (meaning that signals are small compared to measurement noise) but also may be highly variable in time and space in localised coastal and estuarine regions. Spatial variations in trace elements compositions and bioavailablilties do not appear to be spatially hierarchical, and the geographic scale over which detectable chemical contrasts or gradients exist is highly variable among elements and regions. **Consequently, designing a reference database that can capture spatial and temporal variation in elemental concentrations of fish tissues expected in a single site can be challenging.**

Any reference database should contain samples drawn across a suitable spatial area to identify chemical variability within the site. Temporal variations in element availability may occur at high resolution (e.g. rainfall-related differences in freshwater run-off or complexing agents, anthropogenic release) or lower resolution such as pollution mitigation measures. Temporal variations in physiology may also impact the quality of a reference dataset, particularly if the reference database is composed of taxa other than the product in question. Drawing a reference dataset from non-target taxa may, however be necessary in the case of migratory species. Figures 3, 4 and 5 below show how trace element location tests can be created.

Figure 3: Simplest application of trace element methods: testing whether a claimed origin is inconsistent with a single characterised site. MV stands for multivariate axis A and B, one would normally use some multivariate data reduction method to reduce the multiple trace element concentration variables into a smaller number of multivariate axes.
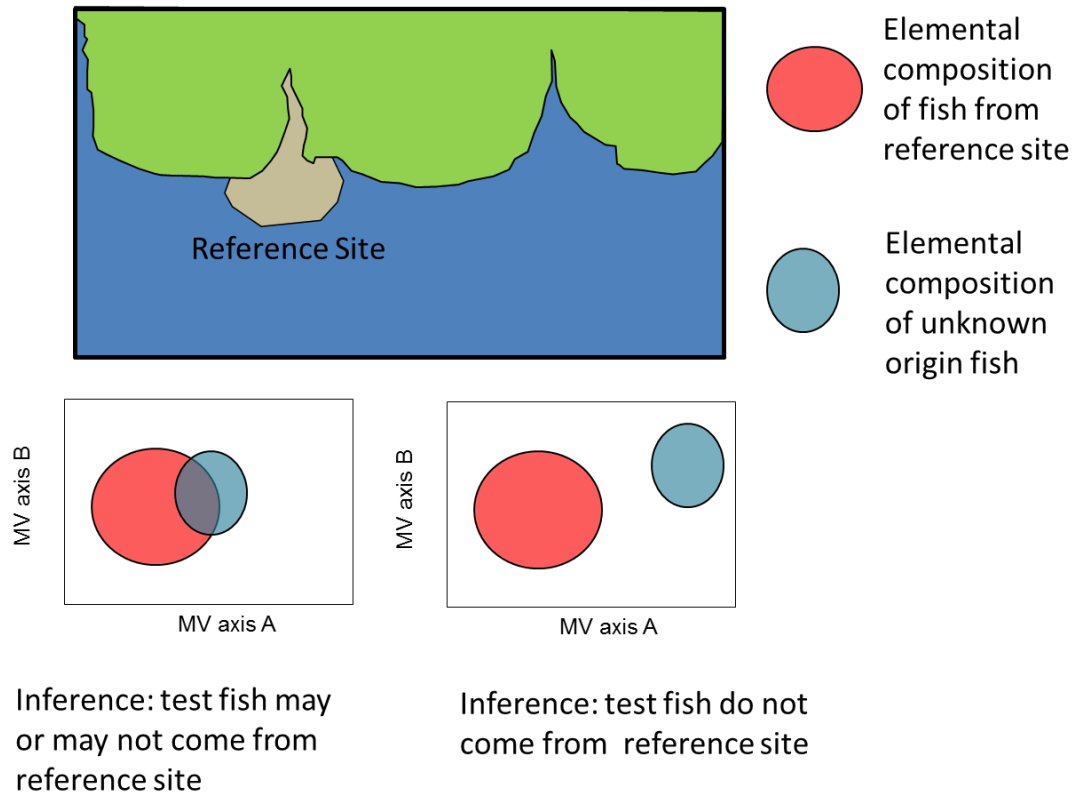
Figure 4: Reference data: requirement for proceeding to use trace element (or any other natural tracer)

Reference Site 1   Reference Site 2

Elemental composition of fish from reference site 1

Elemental composition of fish from reference site 2

MV axis B

MV axis A

MV axis B

MV axis A

Limited potential for trace-element based assignment

Strong potential for trace-element based assignment
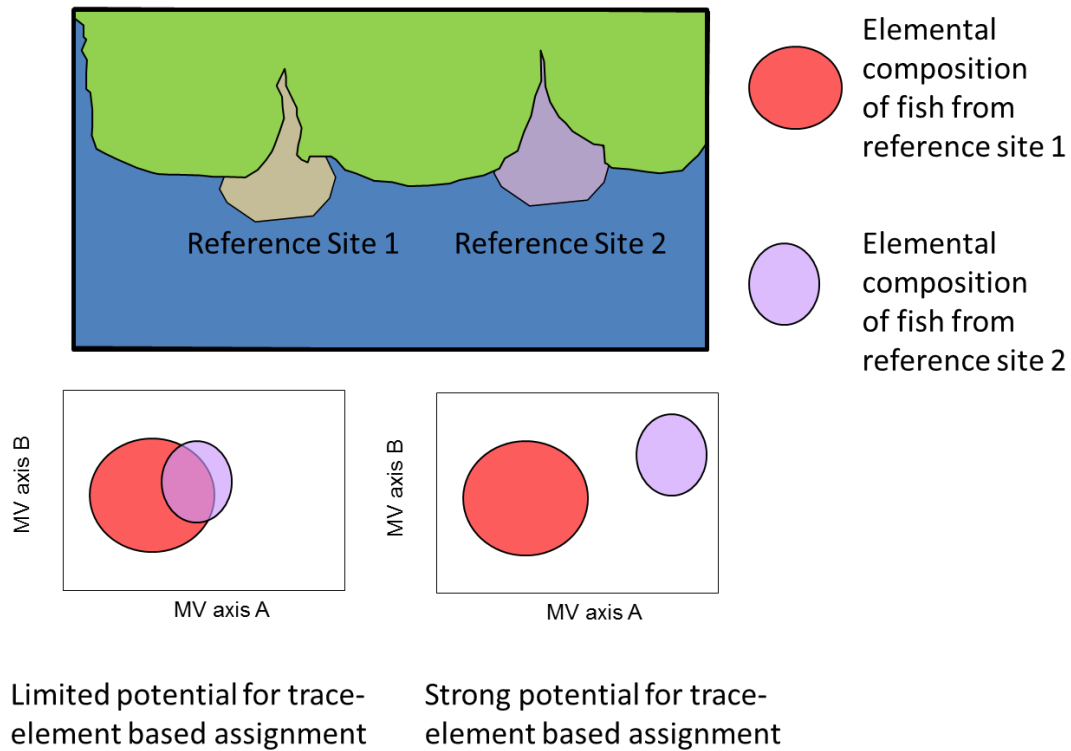
Figure 5: Discriminating between / among discrete characterised sites

Reference Site 1   Reference Site 2

Elemental composition of fish from reference site 1

Elemental composition of fish from reference site 2

Elemental composition of unknown origin fish

MV axis B

MV axis A

MV axis B

MV axis A

Test population does
Not come from site B,
may come from site 1
OR other un-analysed site(s)

Test population may move
between sites 1 and 2, or
may come from other
un-analysed site(s)

3.3 Considerations for reference dataset design

What is the exact structure of the application test?

- *Testing for incompatibility with a single source*
  e.g.: testing whether a suspect product is inconsistent with a single claimed site such as a geographically discrete aquaculture facility (Figure 3). This is the simplest case and where trace element based geolocation is perhaps best suited.

- *Testing between exclusive, spatially defined, locally discrete sites*
  e.g.: establishing the most likely origin among a number of defined sites, ideally that represent all possible origin sites, for instance identifying origin of shellfish among farms or known discrete bays. (Figure 5). Here the potential for successful assignment to origin depends on the chemical separation among sites, and the requirements for characterisation of reference sites is clearly greater than in the single site case above. An excellent example of this approach is provided by Flem et al., (2015) who achieved high level of chemical discrimination between hatchery salmon from 18 farms distributed throughout the Norwegian coastline based on analyses of 12 trace elements in the apatite of the fish's scales.

- *Testing origin between broadly defined geographic regions*
  e.g.: estimating origin of mobile fishes among fishery management zones. This is a very challenging application for trace element based applications as there is limited potential for finding spatially discrete chemical fingerprints between regions, and the practical implications of building a suitable suite of reference data are daunting.

Geographic considerations

The exact spatial area of sites to be compared.
The physical boundaries of the regions to be characterised and tested should be explicitly defined before designing sample collections. This is very simple in an aquaculture application, and relatively simple where the application is focussed on resident and spatially defined population (e.g. Poole Bay clams). Beyond these simple examples, the extent of the region may be defined by a management application such as a fishery region or MPA zoning, or by a hydrological or geographic feature such as depth contours, salinity or current regimes. Management boundaries generally do not coincide with chemical or biochemical gradients. **All natural tracers (e.g. trace elements, fatty acids, isotopes, genetics) are better able to distinguish between animals from environmentally or biologically defined groupings than among political or management units.**
Having decided and defined the spatial extent of the region or regions to be characterised the potential for chemical heterogeneity within and among units must be considered:

Freshwater influences and hydrology - In a coastal marine context, salinity gradients or contrasts are likely to impart the strongest differentiation in bioavailable trace element contrasts within and among regions. Salinity if often well known or modelled

within and among regions. Reference samples must be designed to capture the full range of salinities likely to be encountered among and within regions. In the case of mobile reference organisms, sufficient samples must be taken from a range of locations within the area to maximise the potential of including locally resident individuals. Temporal variations in salinity and the spatial variation in salinity among and between regions should be considered in sample design.

The geological context of watersheds draining into the sites
Associated with salinity, the nature and location of freshwater inputs to the region and the geological context of the watershed can be used to predict potential for chemical discrimination between regions (see section 4). Samples used to define reference datasets must capture the full range of freshwater inputs to the area.

Anthropogenic influences
Urban or industrial diffuse or point source inputs to the water can provide useful identifying trace element fingerprints, but these may be sporadically present and inconsistently distributed within a broad region. Consequently if major urban or industrial inputs are present, particular care in sample design must be placed on the temporal stability and local spatial distributions of trace element concentrations related to anthropogenic inputs.

Seasonal variations and longer term temporal variations
There are two temporal decisions to be made when designing reference datasets: Over what temporal timescale should reference samples be taken, and how frequently do reference databases need to be updated?

The suitable temporal spread of samples comprising a dataset depends on the application and the tissue being analysed. Seasonal variations in salinity, freshwater inputs, water column structure and potentially human inputs are likely to be more important in more inshore, estuarine settings. Reference datasets and test samples should be matched to season.

Longer-term variations in trace element fingerprints are difficult to predict. However, if the principle cause of elemental discrimination among sites is watershed geology, then signals are likely to be relatively temporally stable providing no (a) additional anthropogenic inputs are added and (b) in the case of mobile species, movement ecology does not change.

Taxonomic considerations
Reference databases will be most reliable when constructed using the same species as the test species. In the context of aquaculture sites or sessile invertebrates, this is simple. It is considerably more challenging in mobile species, where obtaining sufficient samples of a test taxon to adequately address the spatial and temporal considerations above may be prohibitively expensive. Using reference data from a model species (such as a sessile invertebrate) may be acceptable if the relationship between the tissue composition of the reference species and that of the test taxon has been tested and shown to be consistent (within quantified uncertainty) over the spatial and temporal range relevant to the study.

Physiological / behavioural considerations

Finally, the reference database should be matched (or at least, not systematically different to) to the test samples in terms of physiological status. Particularly reference and test populations should not differ significantly in age and growth distributions (i.e. do not compare adult and juvenile life stages), reproductive status and sex. In the context of regions with high metal loadings or fluctuating salinity, local adaptation to chemical conditions should be considered as a possible additional source of variance between reference and test populations.

Tissues for testing.
For teleost fishes, otoliths may be preferable because of large literature and confidence surrounding relationship between tissue and water trace element concentrations. Outer regions of an otolith represents the most recent conditions, and modern microsampling allows targeting of very small otolith volumes (µm of otolith material) representing small time periods. Otoliths may not be available if testing is to be performed on filleted samples (or shellfish or elasmobranch fishes). White muscle or liver may be next most suitable target organs. In any case a reference dataset assembled from the tissue to be tested is needed. Fish scales are a potential target tissue containing mineral (calcium phosphate) and organic (collagen) phases, and scales have been used successfully in traceability studies in aquacultured salmonids (Adey et al., 2009, Flem et al., 2017). However, scales are also likely to be absent from processed products. A reference dataset is likely easier to constrain in a closed system such as an aquaculture facility than an open water fishery region.

3.4 Sample size required to characterise a reference sample

Given the discussion above, estimating the sample size required to characterise a reference sample is extremely difficult, especially without pre-existing data. The required sample size will increase with increasing spatial and temporal heterogeneity within each reference region, and decrease as the chemical separation among regions increases. Because the scale of spatial and temporal variation in trace element compositions is highly site and species specific there can be no specific *a priori* guidance on absolute sample numbers needed. Further, the costs associated with building a reference dataset are high, and the risk attached to building datasets that turn out to have limited chemical potential to discriminate among sites is high. Therefore each study needs to be guided by desk-based considerations of the hydrodynamic setting and any pre-existing geochemical data (see section 4).

The sampling requirements associated with building a suitable reference dataset increase with the number of test sites and the complexity of the physical environment.

3.5 Study design: Accuracy and precision and statistical methods

Many statistical methods have been used to compare an unknown sample to a reference database. The simplest approach again lies in the case of testing for incompatibility between a test sample and a reference from a spatio-temporally homogenous source (e.g. where a marketed sample is suspected of falsely claiming provenance from a single aquaculture site). Here the process of analysis follows:

(1) Assembly of the reference samples, calculation of the distributions of each element in the study within the reference sample
(2) Assessment of the measurement error associated with analysis
(3) Assessment of the covariance between concentrations of elements and potentially measurement errors within the sample
(4) Calculation of the likelihood of origin based on (e.g.) multivariate probability distribution functions, considering covariance.

Assessing the accuracy of assignment success in this case is more problematic, given that the approach does not test whether a sample IS derived from the reference population, but rather whether it is inconsistent. One can assess the level of false negative assignments -but this should be negligible or effectively the reference sample is inadequate. Similarly the level of false positive assignments will be dependent on the relative chemical similarity between the reference and test samples, and therefore a function of the choice of locations from which a test sample is drawn. There is therefore no true accuracy value associated with this comparative approach, but as databases grow, the relative proportion of true, false negative and false positive assignments becomes more informative.

A similar analysis chain underpins the case of testing the likelihood of origin from a spatially heterogeneous site, but in this case covariance between elements is likely to increase reflecting (for instance) a common control of salinity on the concentration of several elements.

Several classification approaches can and have been used to distribute the likelihood of origin amongst one of a number of discrete sources. Assessing accuracy and precision has two components: Assessing the degree of separation amongst reference populations and assessing the classification accuracy.

Initial analysis steps remain the same:
(1) Assembly of the reference samples, calculation of the distributions of each element in the study within each reference sample
(2) Assessment of the measurement error associated with analysis
(3) Assessment of the covariance between concentrations of elements (and potentially measurement errors) within the complete sample suite

    The final step is the classification step
(4) Assessment of the likelihood of occurrence from each possible source given the total variance and covariance within and among the reference and test populations

Classification may be performed using multivariate classification tools such as discriminant analyses, random forest, or naïve Bayes classification (e.g. Flem et al., 2017). Assessment of accuracy is relatively simple given a suitable (ideally independent) reference and test dataset. Note that using a resampling tool such as jacknifing to estimate the success of discrimination among reference data will tend to over-estimate the accuracy of assigning an unknown sample, as there may be unrecognised sampling biases contained within the reference datasets. Again as reference databases grow, the confidence associated with classification algorithms and their estimated accuracy will increase.

4. Modelling approaches and improving the technology readiness level

4.1 Overview

As discussed above, while trace element methods offer significant potential for spatial differentiation between sites, the main barrier to the routine application of trace element approaches is the difficulty of estimating the potential accuracy or precision before conducting a pilot study. Designing reference datasets is challenging in all applications other than definition of the trace element composition associated with single-site aquaculture facilities. The technology readiness level associated with trace element based techniques is therefore constrained by the lack of appropriate reference datasets, which is itself limited by the risk associated with committing resources to collecting data that may not reveal suitable geochemical differentiation.

4.2 Suggested model structures

A priori prediction of expected differences in trace element compositions would reduce the risk associated with committing resources to a potentially risky characterisation exercise. At a pure qualitative level, there is an expectation that major differences in baseline geology will be reflected at least in inshore / coastal fisheries. Regions with a historic legacy of metal extraction and associated environmental pollution, with sewage dumping or dumping of dredged sediment frequently display relatively high concentrations of soft acids such as Pb, Zn, Cu Cd in environmental waters and associated fauna.

By contrast, waters in coastal regions draining sedimentary carbonates are typically high in hard metal acid ions such as the essential metals, Sr and Yttrium (Y)., but detailed regional studies of otolith and scale chemistry show that fine scale geographic separation is possible in freshwater, estuarine and shallow marine fishes in UK and Norwegian waters from trace element compositions (e.g. Geffen et al., 2003, Ramsay et al., 2015: Flem et al., 2017).

Compilations of toxic metal tests in fish muscle tissues do not show clear significant regional trends (Rainbow 2018), but these studies span a range of time periods and are typically not designed to test for regional variation. Prediction of the compositions of selected trace metals expected in fish tissues associated with specific geographic regions should be possible – at least at a semi-quantitative level (e.g. predicting 'high', 'medium' or 'low' values), and this may be the most cost effective way to accelerate the technology readiness level of the methodology.

Modelling approaches would be initially informed by spatial maps of geological variation in drainages, local anthropogenic inputs to freshwaters and transfer functions linking likely water compositions to the interaction of geology, topology and land use to predict freshwater inputs to the coastal zone. Appropriate model frameworks have been established to predict strontium isotope variability at a catchment scales in Alaska and used to track movements of salmon (Brennan et al., 2015). Subsequently hydrological models of coastal environments could be drawn on to predict the spatial extent and spread of freshwater metal input within the coastal

zone, and coupled with data on the historic and current levels of industrial and sewage waste disposal in the region.

It is unlikely that such a modelling framework could predict relative metal concentrations or bioavailablilties in a fully quantitative fashion, but semi-quantitative or conditional model parameterisations such as Bayesian Belief Networks (BBN) are an attractive option for prediction of the likelihood of measurable differences in trace element compositions between locations. BBN approaches are especially attractive in this context due to their ability to be updated based on new information (e.g. development of reference datasets). A BBN model predicting spatial differences in trace element concentrations of fish tissues would therefore increase in accuracy with continued use, and would also provide a desk-based risk assessment ahead of committing to costly design of reference sampling.

4.3 Developing reference datasets

In parallel with model development, TRLs would be enhanced by development of a systematic sampling program to build spatial reference datasets based on a common suite of species and tissues analysed under controlled and uniform conditions. Such databases could initially be focussed on restricted, hydrologically simple regions with commercial significance, and would initially assist in validation and improvement of models, but would in time be sufficient to act as reference datasets in traceability studies. An example exercise, although much larger in scope, is conducted by the British Geological Survey (the Geochemical Baseline Survey of the Environment -G-BASE. The G-BASE project builds a reference dataset of the geochemical composition of soils and groundwater across the UK. The initial outlay in resources was extensive, but the dataset now underpins a wide variety of planning, environmental and prospecting applications and is a source of revenue for the BGS. G-BASE also provides data that could be used in any modelling framework predicting spatial variation in estuarine and coastal elemental compositions in fish tissues. Linked modelling and database development programs have the potential to significantly increase the technology readiness level of trace element based traceability applications. Without such investments, studies are likely to remain small in scale, tailored around specific geographic or commercial applications, and comparison across studies will be problematic due to additional variance associated with the choice of samples, sampling design and analytical methods used.

5. Example costed study considerations

Indicative costs based on realistic sampling requirements for an applied (as opposed to a pilot or academic exercise):

Scenario A – Specific and spatially discrete site

Characterising a multivariate trace element fingerprint for a specific and spatially discrete site (e.g. fixed location aquaculture facility or spatially restricted shellfish fishery).

At the most basic level a sample size of 50-100 individuals sampled from each of any replicated cages or shellfish beds, representing the range of body sizes at harvest and drawn from a random sample of individuals repeated though the harvest period. For a facility harvesting throughout the year this might represent a minimum of 4 (seasonally separated) sample periods, potentially nesting samples over 5-10 days within each seasonal sample to capture short-term variability.

Minimum sample size for single cage facility with no *a priori* expectation for temporal variation in water chemistry other than seasonal fluctuations should consider 200 individuals.

Analysis costs for a broad spectrum trace elements vary widely depending on the access to facility and commercial vs in house analysis costs. In house, economic cost analysis costs approximate on the order of £500-£1000 per day analysis time, which equates to c. £5-£10 per sample. Therefore a minimum indicative cost for the analysis of a small reference sample for the simplest possible application is c. £1000-£2000 excluding any personnel costs.

Scenario B – A number of specific and spatially discrete sites

Characterising multivariate trace element fingerprints for a number of specific and spatially discrete sites

At a basic level the indicative cost associated with scenario A could be multiplied by the number of sites to be compared, but by increasing the number of sites it is likely that additional local factors will be introduced (e.g. sites containing salinity gradients or point sources of anthropogenic inputs), and the sampling requirements are therefore likely to increase faster than the number of sites to be assessed, depending on the geography and hydrology of the sites.

Scenario C – Open Water

Open water sites and migratory species

This is a challenging scenario to design reference samples. Most available samples are likely to be spatially and temporally biased, and therefore to under-represent true variance within a region. Particular care should be taken to avoid pseudo-replication rising from sampling multiple individuals from a single catch chosen to represent individuals within a region. Inevitably costs associated with building a reference

population increase dramatically with mobile offshore species. There is considerable danger associated in assuming that opportunistically sampled individuals drawn from catches are representative of the full elemental variability potential within a defined region. As the size or geographic/hydrological complexity of the region increases, the risk associated with under-sampling clearly increases. For this reason, trace element techniques are poorly suited to characterising catch location in open water and especially highly mobile taxa.

6. Summary

Trace element based approaches are attractive because of large number of variables and potential for discriminatory power.

Trace element based spatial approaches have proven successful in elemental discrimination among spatially separated stocks using otolith and scale markers and also in terrestrial foodstuffs, especially in combination with isotopic approaches.

The suite of elements that can be used is limited by the level of physiological control of many elements in fishes, the relative homogeneity of marine waters, and the cost of assembling suitable reference samples.

Spatial variation in bioavailable trace element compositions tends to occur at local, small spatial scales rather than broad regional scales, and in the case of elements such as Cd, Cu, Zn, Pb, As and Hg largely reflects current and historic anthropogenic inputs to the marine environment.

Combining trace element with stable isotope, genetic and other natural tracer methods can make best use of the different geographic scales underpinning spatial gradients in natural tracers. In coastal and especially inshore and estuarine regions, trace element tracers may provide fine scale differentiation that cannot be obtained using any other natural tracer.

Trace element based approaches are perhaps most immediately suitable for characterisation of discrete source aquaculture facilities or similar localised shellfish production sites.

Technological readiness level is best increased through the development of a modelling capacity to predict likely differences in trace element compositions among target sites, and a complimentary development of reference datasets.

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