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Trace elemental fingerprinting of shells and soft tissues can identify the time of blue mussel (*Mytilus edulis*) harvesting

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ABSTRACT

Reliance on seafood for a source of animal protein is growing globally and this is likely to continue as Earth's population continues to rise. An active shift towards farmed produce over wild caught is occurring, attributed to dwindling wild populations, increased productivity potential and increased food security needs. Although production is rising, producers and regulators are continually challenged as passive filter feeding shellfish such as mussels are impacted by disease outbreaks, toxic algae blooms, pollution and food fraud that pose a risk to the market. This risk can manifest as mortality events and loss of stock, but also via consumer safety and subsequent loss of trust. To combat this threat, accurate and reliable traceability tools are necessary to give regulators power to maintain consumer safety and subsequently, trust. Recent research has demonstrated that trace element fingerprints (TEFs) based on the shell and soft tissues can identify the site of harvest of blue mussels (*Mytilus* edulis) (Bennion et al., 2019. Trace element fingerprinting of blue mussel (*Mytilus edulis*) shells and soft tissues successfully reveals harvesting locations. *Science of The Total Environment,* 685, 50–58) and king scallops (*Pecten* maximus) (Morrison et al., 2019. Spatio-temporal trace element fingerprinting of king scallops (*Pecten maximus*) reveals harvesting period and location. *Science of The Total Environment,* 697, 134121) with 100% success. Here, we test the temporal stability of trace element fingerprints (TEFs) of blue mussels within the aquaculture sphere, over five harvesting dates spanning two years. Computational models constructed using the trace element signatures of shells and soft tissues show near absolute temporal differences of TEFs between harvesting dates. However, TEFs based on a combination of both the shell and periostracum of mussels enabled 96% of all individuals to be correctly assigned to their date of harvest indicating that this method can not only identify the location but also the date of harvest of bivalve shellfish. This technique offers a reliable scientific-based traceability tool for regulators to uphold food safety standards and can prove an invaluable asset within the seafood regulatory arsenal.

1. Introduction

According to FAO, 2018, bivalve aquaculture typically accounts for between 14 and 16% of average per capita animal protein for 1.5 billion people. The vast majority of bivalve production throughout the world is now farmed (~89%) FAO, 2016, following a major shift in seafood production in recent years. This upward trend in aquaculture production is predicted to increase further (Anderson, 2002; Bostock et al., 2010). Already molluscan aquaculture accounts for 21.42% of total farmed seafood production, with Asia being the greatest contributor (Soon & Ransangan, 2019, FAO, 2018). Aside from a significant source of animal protein, bivalve aquaculture also supports livelihoods for upwards of 200,000 people in mainly job-poor regions throughout the globe (FAO, 2018).

One of the main limiting factors to farmed bivalve productivity is disease outbreak (Rowley et al., 2014; Stentiford et al., 2012) and the occurrence of mass mortalities owed to a combination of intrinsic (e.g. disease) and extrinsic factors (e.g. water temperature & depletion of dissolved oxygen) (Soon & Ransangan, 2019). These issues are receiving significant attention globally, in an effort to mitigate losses (Behringer

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et al., 2020; Stentiford et al., 2017). Receiving less attention, are issues surrounding food fraud and counterfeit produce (Jennings et al., 2016; Miller et al., 2012). Recently, a spotlight has been placed on seafood fraud owed to the efforts of studies like Warner et al. (2013) from the United States, Hanner et al. (2011) from Canada and Lamendin et al. (2015) from Australia. Advances in the field of DNA metabarcoding allowed researchers and investigators in these cases to quickly and cheaply genetically identify the species in processed seafood, illuminating the scale of food fraud within the market. What these studies, and many others have shown (e.g. Warner et al., 2013; Warner et al., 2016), is that the issue of food fraud is significant in the marketplace although scale is regionally dependent (Jennings et al., 2016). Counterfeit produce and mislabelling pose three main threats. Firstly, food fraud can pose an indirect risk to the market, whereupon consumers can lose faith in producers and industry regulators (Gordoa et al., 2017; Kroetz et al., 2018). Secondly, there is a threat to producers and animals outside of regulated systems. For example, seafood that is obtained from illegal, unreported and unregulated (IUU) fishing or other unregulated systems where stocks are not appropriately monitored or managed and workers are not adequately protected (Fox et al., 2018; Pramod et al., 2014). In the US for example, between 20 and 32% of wild caught produce imported into the country in 2011 was estimated to be of an 'IUU' source (Pramod et al., 2014). Thirdly, food fraud can pose a health and safety risk to consumers who unknowingly consume inferior or counterfeit produce (Fox et al., 2018; Warner et al., 2013). Counterfeit produce may not have passed rigorous quality control checks that are in place to protect consumers (Warner et al., 2013).

Health risks associated with consuming inferior shellfish produce are significant (Fox et al., 2018; Karunasagar, 2008). Harmful algae blooms (HABs) comprising of biotoxin producing microalgae can pose potentially life threatening risks to consumers if shellfish which have filter-fed on these planktonic algae are consumed (Hallegraeff & Bolch, 2016; Landsberg, 2002; Shumway, 1990). The oceans also provide a medium for vector-free transmission of potential pathogens, some of which can also pose a risk to consumers if contaminated produce are eaten (e.g. *Vibrio* spp.) (Hsern Malcolm et al., 2015; Xie et al., 2016). Again, owed to the sedentary life history of many bivalves, and their passive feeding mechanisms, they have the potential to accumulate trace elements and organic pollutants at potentially hazardous concentrations (reviewed by Guéguen et al., 2011). Fortunately, the incidence of contaminated produce making it to market is rare, as is the onset of bivalve associated illness in consumers (O'Mahony, 2018; EFSA, 2018). Nevertheless, regular harvesting closures owed to poor water quality can have broad and complex socio-economic implications for receiving communities (Evans et al., 2016). Yet due to the rapid expansion and acceleration of the bivalve aquaculture industry, it is likely that monetary incentive will entice efforts of food fraud going forward. Industry regulators currently have checks and precautions in place to combat food fraud but no standardised scientifically based tool is presently available that allows regulators to trace molluscan produce back to its source.

Recognising a need for accurate provencing tools, work in the field of seafood traceability is accelerating (Warner et al., 2013; Leal et al., 2015; Bailey et al., 2016; Ricardo, Pimentel, Génio, & Calado, 2017; El Sheikha & Xu, 2017; Milan et al., 2019; Bennion et al., 2019; Morrison et al., 2019). The use of genetic tools has shown promise in some situations (location, taxa and rearing method specific) (Martinsohn et al., 2019; Milan et al., 2019). Issues surrounding the spatial resolution of DNA-based techniques have limited its wide-scale application thus far. Martinsohn et al. (2019) postulate that misconceptions regarding costs associated with molecular techniques have stunted its use in the seafood traceability sphere. Studies based on stable-isotope and fatty acid analyses have demonstrated some potential as scientifically based traceability tools (Camin et al., 2016; Carter et al., 2015; Gong et al., 2018; Gopi et al., 2019; Ricardo et al., 2015b, 2017b). However, it remains to be seen how these tools could be applied in a standardised manner to trace produce, regardless of taxa, locale and rearing method. Supply chains are typically complicated in the seafood sector with processors tending to handle both farmed produce from multiple sources and wild caught produce. Moreover, practices within the aquaculture industry can significantly frustrate traceability efforts. For example, most of the spat for New Zealand's green-lipped mussel aquaculture industry is wild sourced from one beach in New Zealand's Far North (Dunphy et al., 2011) with seed origin locations unknown. Shellfish seed is also traded and selectively bred worldwide (e.g. in Europe) (Chavanne et al., 2016). These diverse sourcing systems complicate the task of creating a traceability tool that can be applied to all cultured populations regardless of where and how spat/seed has been sourced. Supply chain factors such as these introduce significant complexity in terms of traceability, impacting the viability of some scientifically based traceability tools. For example, molecular tools at the population level are limited due to relatively few hatcheries supplying spat to large numbers of producers within the bivalve aquaculture sector.

For these reasons, the use of trace element fingerprints has been identified as a likely 'best fit' and 'one-size-fits-all' traceability tool for molluscan produce (Bennion et al., 2019; Morrison et al., 2019). Numerous published studies have now shown considerable potential of both laser ablation and plasma mass-spectrometry based trace elemental fingerprinting or 'TEF' of bivalve shells to correctly assign bivalve molluscs to their respective harvesting/rearing location (Bennion et al., 2019; Dunphy et al., 2015; Morrison et al., 2019; Ricardo et al., 2015a, 2017a; Zhao et al., 2019). Recent research has demonstrated that trace elemental fingerprints (TEFs) based on a combinations of shells and soft tissues can correctly identify the harvesting location of both blue mussel (*Mytilus edulis*) (Bennion et al., 2019) and king scallops (*Pecten maximus*) (Morrison et al., 2019) with 100% success, including between two sites just 6 km apart within a single bay in the case of blue mussels. Ricardo, Pimentel, Génio, and Calado (2017), suggests a 'reference library' of trace element fingerprints would be needed for such a TEF tool to be implemented but importantly, that the usefulness of any traceability tool lies in its ability to predict not just correctly categorise individuals spatially, but also whether such site specific TEFs are temporally stable. Morrison and co-authors (2019) demonstrated that not only were the TEFs of the shell and adductor muscle of king scallops not temporally stable, but that this method could identify individuals with 100% success between two harvesting dates just 6 weeks apart from the same location. While such temporal variability means that a reference library of all production sites cannot be based on a 'once-off' determination of TEFs, instead such a library would need to be updated periodically. However, there is an obvious significant benefit of a TEF based traceability tool that can identify not just the site of harvest but also the time frame of harvest.

Therefore, an understanding of how often this 'library' would need to be updated, to provide ongoing confidence in predictions made is required. Building on the work of Bennion et al. (2019) and Morrison et al. (2019), and following the important points made by Ricardo, Pimentel, Génio, and Calado (2017) surrounding the creation of a reference library of trace element fingerprints, the present study strives to 'fill-in' some of these remaining unknowns, facilitating the creation of a scientifically-based traceability tool for molluscan produce. Here, we make use of the micro-elemental concentrations of blue mussel (*M. edulis*) shells, soft tissues and secretions, collected on five dates between January 2016 and January 2018 to investigate the temporal stability/variability of this TEF-based traceability tool.

2. Methods

2.1. Specimen collection

Ten *M. edulis* specimens with shell length >40 mm (marketable size) were collected from one single site, Killary Fjord (Longitude: 53°37′11.8″N Latitude: 9°50′45.2″W) on five different occasions, January 2016, January 2017, May 2017, September 2017 and January

2018 ($n = 50$) (Fig. 1). The geology in the immediate area is sandstone, conglomerate and mudrocks. The catchment surrounding Killary Harbour fjord primarily consists of peat bogs, small areas of conifer plantation and natural grassland.

2.2. Experimental design and specimen processing

The methodology used here is described in detail by Bennion et al. (2019) and is similar to previous studies that used ICP-MS determined TEFs to identify the harvesting location of cockles, *Cerastoderma edule* (Ricardo et al., 2015a, 2017a), goose barnacles, *Pollicipes pollicipes* (Albuquerque et al., 2016) and king scallops, *Pecten maximus* (Morrison et al., 2019). Briefly, all laboratory equipment used for specimen preparation for elemental analysis were soaked in 10% HNO_3 overnight, triple rinsed with ultrapure H_20 (18.2 mΩ, Milli-Q Element System™, Merck Millipore, USA). Valves were separated and the foot was removed using acid soaked plastic forceps and ceramic blades. Removed tissues were stored frozen (−20 °C) until further processing.

Before acid digestion, the left valve (referred to as "clean shell" from here onward) was soaked overnight (14 h) in 15% trace analysis grade H_2O_2 [TraceSelect® Ultra, Sigma-Aldrich, St. Louis, USA] and triple rinsed with ultrapure water to remove the periostracum and any foreign matter. The periostracum was removed from the clean shell was retained for elemental analysis. The periostracum is typically removed (and disposed of) prior to analysis of shell microchemistry as it has a quick tissue turnover rate and therefore, a more rapid uptake of bioavailable elements in the environment in comparison to the slower forming calcareous shell (Bellotto & Miekeley, 2007; Szefer et al., 2002). This fast turnover rate has the potential to impact provencing success (Bennion et al., 2019). Though this cleaning process may also impact results by manipulating the trace element signatures available for TEF (Bennion et al., 2019). For this reason, right valves (referred to as "unclean shell" from here onward) were briefly cleaned via rinsing with ultrapure H_20 and scrubbed with an acid soaked (10% HNO_3) nylon

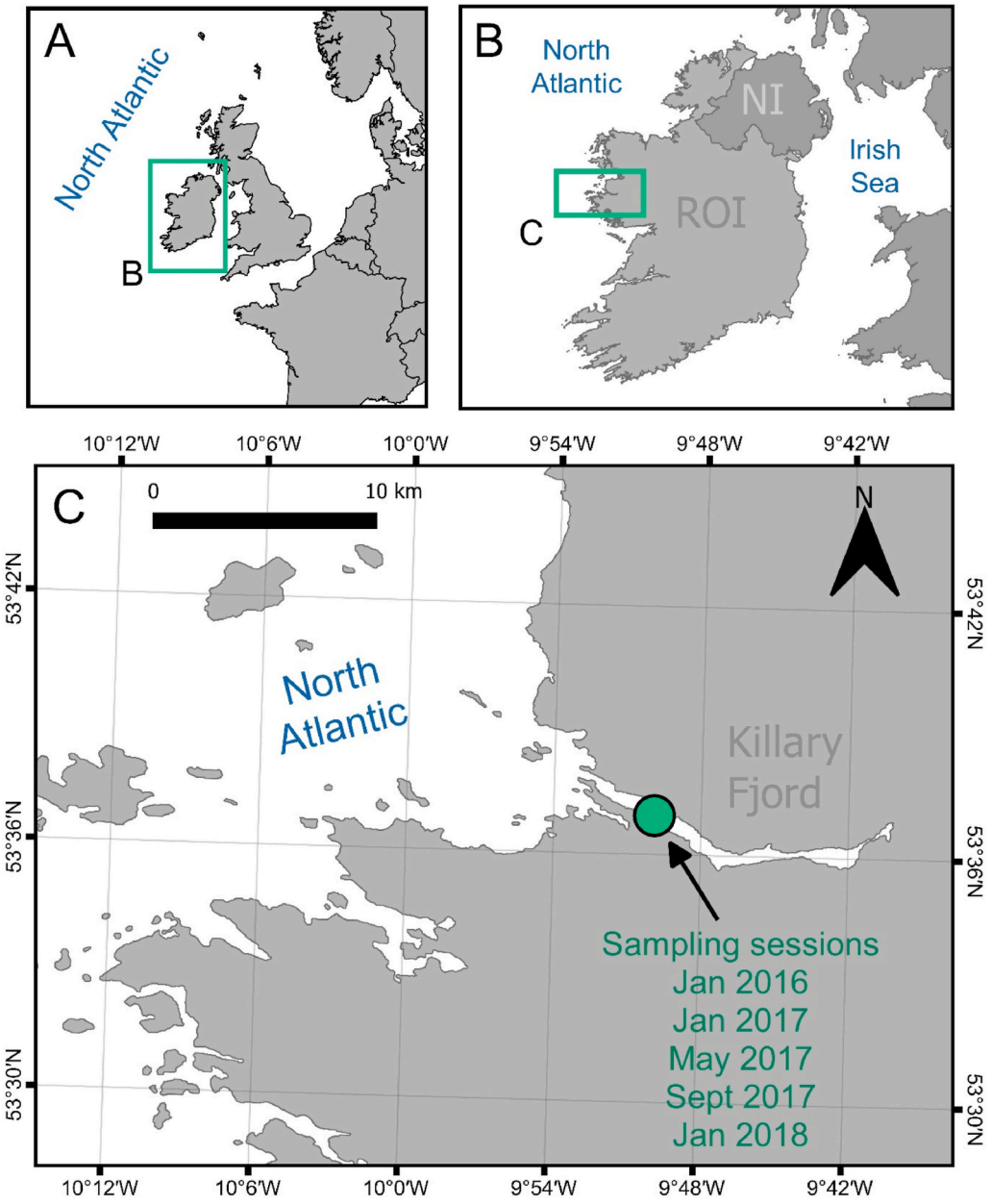

Fig. 1. Sampling location and dates of harvest sessions of blue mussels (*M. edulis*) from the from outer Killary Fjord on the West coast of Ireland. Map created using Quantum GIS. Spatial data obtained from DIVA-GIS. CRS: WGS 84 (EPSG: 4326).

brush to remove any foreign matter. The periostracum was left intact in order to examine if trace element fingerprinting success of mussels to their respective harvesting locations differs when shells are cleaned via typical cleaning methods and left 'uncleaned'.

The most recently formed part of the shell, after the last growth annuli (the latest year of growth), was removed from the posterior end of each valve ('clean' and 'unclean') (Fig. S1). To remove the latest growth area, shells were air dried for 12 h following the cleaning procedure (i.e. nailbrush or H_20_2). The drying of the shells allowed for easy detection of growth bands visually (McGrorty, Clarke, Reading, & Goss-Custard, 1990; Sukhotin et al., 2002). Using acid washed plastic tweezers and a ceramic blade, the shell (after the last growth band to the shell posterior edge) was chipped away, using the ceramic blade if necessary, to aid removal and avoid splintering the shell (Fig. S1). The entire foot was removed and following acid washing of the "clean shell" the periostracum was peeled off the shell using acid washed plastic tweezers. Foot and periostracum tissues were freeze-dried (−52 °C) [Freezone 12, Labconco, Kansas City, MO, USA]. The entire homogenized foot and periostracum from each specimen was acid digested for analysis.

2.3. Acid digestion and chemical analyses

Pre-processed shells and soft tissues (shell fragments, periostracum and foot) were precisely weighed (to the nearest mg) and digested in Teflon™ closed vessels (at 80 °C for 14 h) using ~70% HNO_3 [SpA grade, Romil™, Cambridge, UK] and 30% H_2O_2 [TraceSelect® Ultra, Sigma-Aldrich, St. Louis, USA]. The digestates were diluted to 25 mL with ultrapure water. To control for Ca potentially concealing the concentration of other elements during shell sample analysis, subsequent dilutions were carried out on the digestates of the shell fragments. A large number of elements were examined in this study. This large suite of elements was examined for two reasons i) to increase the number of variables available for model construction, the concentration of which are free to vary temporally and therefore the likelihood of predictive success being achieved and ii) to determine which elements in the shells and soft tissues contribute most to trace element fingerprints. Elemental determinations (^{107}Ag, ^{27}Al, ^{75}As, ^{11}B, ^{138}Ba, ^{9}Be, ^{209}Bi, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{69}Ga, ^{74}Ge, ^{115}In ^{55}Mn, ^{93}Nb, ^{60}Ni, ^{208}Pb, ^{121}Sb, ^{45}Sc, ^{82}Se, ^{118}Sn, ^{130}Te, ^{47}Ti, ^{205}Tl, ^{238}U, ^{51}V, ^{184}W, ^{66}Zn) were performed using an Elan DRC-e Inductively Coupled Plasma–Mass Spectrometry, ICP-MS, [PerkinElmer, USA] in standard mode and ^{52}Cr, ^{56}Fe, ^{82}Se, ^{66}Zn in dynamic reaction cell (DRC) mode with methane as the reaction gas (Healy et al., 2016; Wilkes et al., 2017) in a class 1000 clean room (ISO 6).

2.4. Quality assurance

For analytical validation purposes, Certified Reference Materials (CRM) ERM® -CE278K (wild mussels, *Mytilus edulis*, harvested off the coast of the Netherlands) from the European Commission, Joint Research Centre Institute for Reference Materials and Measurements (IRMM) and alongside method blanks and duplicate samples, were prepared and analysed alongside the samples. The CRM (ERM® -CE278K) provided an identical matrix match with the unknowns and the observed concentrations were in close agreement with the certified values (Table 1).

2.5. Data analyses

Similar to Bennion et al. (2019) and Morrison et al. (2019) to account for variation in the amount of shell and soft tissue analysed by ICP-MS, true elemental concentrations were calculated using the known mass of shells and soft tissues, obtained prior to digestion. Thus, concentrations used in model construction were in the format μg g^{-1}. Prior to data analysis, internal standards were compared to elemental concentrations to identify which, if any, elements that fell below the limit of detection.

Table 1
Observed results from the analysis of the mussel tissue Certified Reference Material ERM® -CE278k (European Commission, Joint Research Centre). All values are in μg g^{-1} (n = 20) (SD = Standard deviation).

Element	ERM® -CE278k certified value (±SD)	Observed this study (±SD)
As	6.7 ± 0.4	5.27 ± 1.16
Cd	0.336 ± 0.025	0.295 ± 0.046
Cr	0.73 ± 0.22	0.661 ± 0.187
Cu	5.98 ± 0.27	6.44 ± 1.67
Fe	161 ± 8	168 ± 47.32
Mn	4.88 ± 0.24	5.29 ± 1.40
Ni	0.69 ± 0.15	0.82 ± 0.24
Pb	2.18 ± 0.18	1.80 ± 0.42
Se	1.62 ± 0.12	1.79 ± 0.39
Zn	71 ± 4	68.22 ± 9.9

Therefore, Be, Ba, Ge, Nb, Ag, Sb, Te, Ti, Bi and Si were removed prior to model construction. Elemental concentrations in the CRM (mussel and fish tissue) were then compared to known concentrations to identify 'percentage recovery'. Elements which were not successfully recovered in the CRMs were also removed from all analyses (Cr and Se).

Similar to both Bennion et al. (2019) and Morrison et al. (2019), Breiman Cutler random forests classification (Breiman, 2001) method, applied using the R package "randomForest" (Liaw & Wiener, 2002) was used to test if blue mussels could be assigned to their respective date of harvest based on their trace elemental fingerprints. This classification process is a bootstrap aggregation method that develops many classification trees using random bootstrapping of the data in which each individual blue mussel is classified to one of the potential harvest dates by applying each of the fitted trees in the generated forest to the observation, with assignment to the date of harvest selected by the majority of trees (Breiman, 2001; Liaw & Wiener, 2002). Using random forest analyses to examine if mussels have distinct temporal trace elemental signatures has considerable advantages over other statistical classification approaches. This statistical approach is robust to overfitting if the number of predictor variables is greater than the number of samples in the dataset and it is also a powerful approach statistically when there are many weak explanatory variables with no single or small subset of variables that can distinguish between classes. Additionally, random forest classification does not require the strict multivariate distributional and normality assumptions required by other discriminant methods (Breiman, 2001).

This random forest classification was performed nine times on the blue mussel (n = 10 per date) elemental concentrations of each of: periostracum, foot, unclean shell, clean shell, as well as the following combinations: periostracum and foot, clean shell and periostracum, clean shell and foot, unclean shell and foot and finally, clean shell, foot and periostracum, which is all possible combinations of the structures and identical to the approach of both Bennion et al. (2019) and Morrison et al. (2019). The analyses using combinations of structures were conducted to increase the number of predictor variables included in the random forest procedure. Each Breiman Cutler random forests classification model was performed using the R package, randomForest (Liaw & Wiener, 2002) and had 1001 classification trees each, with the number of predictor variables randomly selected as candidates in each node limited to the square root of p, where p is the number of predictor variables in the analyses. All models were constructed in R Studio v. 3.4.2 (R Core Team, 2019).

3. Results

Using TEF and random forests modelling, the magnitude of success varied based on the combination of molluscan tissues, structures used, and the time samples were gathered (Table 2 & Fig. 2). The least successful predictions were based on the use of the trace element signatures of individual soft tissues, the periostracum (total correct, 80%) and foot

Table 2
Classification success rates of the random forest models based on trace elemental fingerprints of *M. edulis* determined from the all four tissue types and combination of structures on all sampling dates.

Structure(s)	Collect site	Predicted Collection Site					% correct	Total % correct
		Jan. 2016	Jan. 2017	May 2017	Sept. 2017	Jan. 2018		
Periostracum	Jan. 2016	**10**					100	80
	Jan. 2017		**6**		1	3	60	
	May 2017	1		**9**			90	
	Sept. 2017	1			**9**		90	
	Jan. 2018		3		1	**6**	60	
Foot	Jan. 2016	**10**					100	87.8
	Jan. 2017		**8**	1		1	80	
	May 2017			**10**			100	
	Sept. 2017		1	1	**8**		80	
	Jan. 2018		1	1		**7**	77.8	
Unclean Shell	Jan. 2016	**10**					100	80
	Jan. 2017		**7**	1		2	70	
	May 2017			**9**	1		90	
	Sept. 2017			1	**7**	**2**	70	
	Jan. 2018		2		1	**7**	70	
Clean Shell	Jan. 2016	**10**					100	90
	Jan. 2017		**9**	1			90	
	May 2017			**8**	2		80	
	Sept. 2017		1		**9**		90	
	Jan. 2018			1		**9**	90	
Periostracum & Foot	Jan. 2016	**10**					100	87.8
	Jan. 2017		**9**	1			90	
	May 2017			**10**			100	
	Sept. 2017		1		**9**		90	
	Jan. 2018		2		2	**5**	55.6	
Clean Shell & Periostracum	Jan. 2016	**10**					100	96
	Jan. 2017		**10**				100	
	May 2017			**10**			100	
	Sept. 2017			1	**9**		90	
	Jan. 2018				1	**9**	90	
Clean Shell & Foot	Jan. 2016	**10**					100	95.9
	Jan. 2017		**9**	1			90	
	May 2017			**9**	1		90	
	Sept. 2017				**10**		100	
	Jan. 2018					**9**	100	
Unclean Shell & Foot	Jan. 2016	**10**					100	91.8
	Jan. 2017		**9**	1			90	
	May 2017			**10**			100	
	Sept. 2017			1	**9**		90	
	Jan. 2018				2	**7**	77.8	
Clean Shell, Foot & Periostracum	Jan. 2016	**10**					100	93.9
	Jan. 2017		**9**	1			90	
	May 2017			**10**			100	
	Sept. 2017		1		**9**		90	
	Jan. 2018				1	**8**	88.9	

(total correct, 87.8%). Based on the use of the shells alone the model was able to predict 80% of individuals to correct sampling period using the 'unclean' shell and 90% of individuals using the 'clean' shell (Table 2 & Fig. 2). To improve the likelihood of classification success by increasing the number of variables and thus potential for unique reference chemical signatures, a combination of the TEFs from multiple structures was used to create the model. The combination of the trace element signatures of both the periostracum and foot combined did not improve classification success of the model based on the TEF of the foot alone (total correct, 87.8%). The combination of the trace element signatures of the unclean shell and the foot, improved classification success to 91.8% and the clean shell and the foot combined improved success further to 95.9%, with the combination of the clean shell reference chemical signatures and that of the periostracum proved the most successful classification with 96% of mussels correctly assigned to their date of harvest, with just two individuals misclassified to the previous sampling date. Interestingly, when a model was created using a combination of the reference chemical signatures of the clean shell, foot and periostracum, classification success was reduced to 93.9% (Table 2).

The predictions here are based on several collection sessions rather than sites, beginning in January 2016 and ending in January 2018. For all structures and combinations therein, the model was able to successfully predict all individuals to the January 2016 sampling effort. Following this, the success rate began to decline considerably for the predictions based on single structures, but less so for those based on a combination of chemical signatures from multiple structures (Table 2 & Fig. 2). For example, the model based on the periostracum alone, classified only 60% of individuals correctly to January 2017 (January 2016, 100% correctly classified) and the model based on the foot alone was only marginally better, classifying 70% correctly to January 2017 (Table 2). Illustrated clearly by the total percentage correct, the combination of the TEFs of the clean shell and periostracum provided the greatest success between sampling dates. Using this combination, the model was able to place individuals into correct sampling dates 100% of the time for January 2016, 2017 and May 2017. For September 2017 and January 2018, the model misplaced one individual for each sampling session, reducing overall classification success to 96%.

Variable importance to the random forest analysis for the clean shell and the periostracum (the most successful model) is shown in Fig. 3. Predictive contribution to the model is characterised by mean decrease accuracy in descending order on the y-axis. By far, iron (Fe) and lead (Pb) in the clean shell were the most important predictor variables,

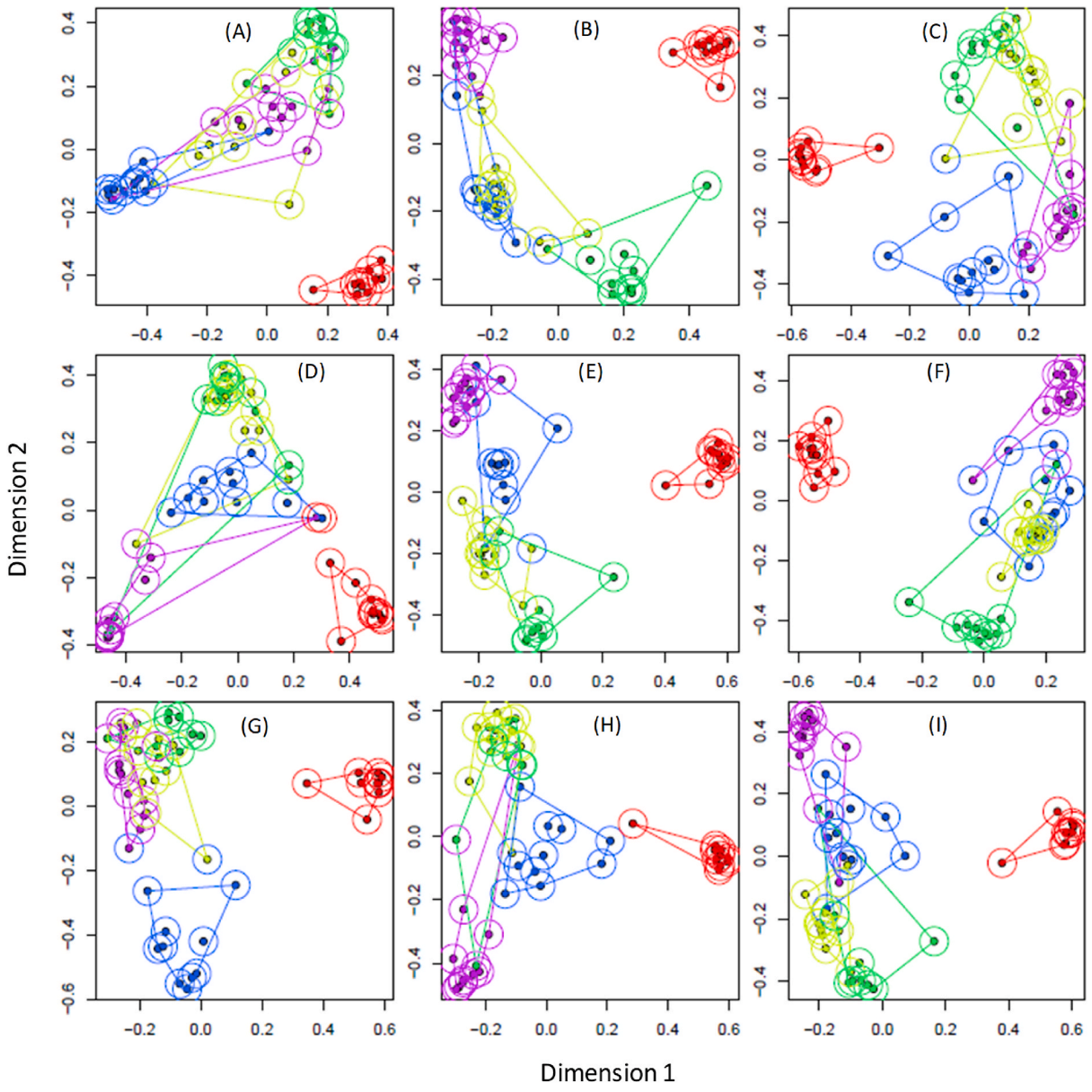

Fig. 2. Multidimensional scaling ordinations of proximity scores from each of the random forest classifications based on the elemental concentrations of (A) unclean shell (B) clean shell (C) foot (D) periostracum (E) clean shell and foot (F) clean shell and periostracum (G) unclean shell and foot (H) periostracum and foot (I) clean shell, foot and periostracum. Convex hull is drawn around the a-priori classes, with points coloured according to the date of collection and outer ring colour refers to the date each individual was classified to according to the random forest analyses. Red = January 2016; Yellow = January 2017; Blue = May 2017; Purple = September 2017; Green = January 2018. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

followed closely by tungsten (W) in the periostracum and cadmium (Cd) in the clean shell. A gradual decline in variable importance follows, with magnesium (Mg) and vanadium (V) from the cleaned shell contributing the least to the predictive success of the model (Fig. 3). A large suite of elements was examined here. The goal of sampling this array of elements, including several which are not typically examined in studies such as this (e.g. V, W, Ge, Ga and Nb), was to increase the likelihood of detecting unique trace element fingerprints, and subsequently increase the model's predictive accuracy. Elements that were not adequately recovered in certified reference materials and those that fell below the limit of detection of the analytical technique were omitted from the data analysis. By not discounting elements for analysis at the earlier stages (chemical analysis), model construction was not constrained by a limited number of pre-selected elements. This exploratory approach was taken to determine which elements were the most important for classifying specimens correctly. In future, depending on the spatial and temporal scales, and the structure(s) analysed, the number of elements could potentially be reduced based on site- and temporally-specific bioavailability.

4. Discussion

The use of random forests models based on trace element fingerprints of structures from *M. edulis* has illustrated the potential of this TEF based tool for traceability of molluscan produce. The temporal variability of TEFs has been shown, evidenced by the varying degrees of prediction success, which was achieved by the model over time. The use of the chemical signatures of the clean shell and periostracum combined, provided the greatest and most consistent classification success rate (96% total classification success). In contrast to work previously carried out by Bennion et al. (2019) and Morrison et al. (2019), the predictions here were based temporally rather than spatially. For that reason, it is

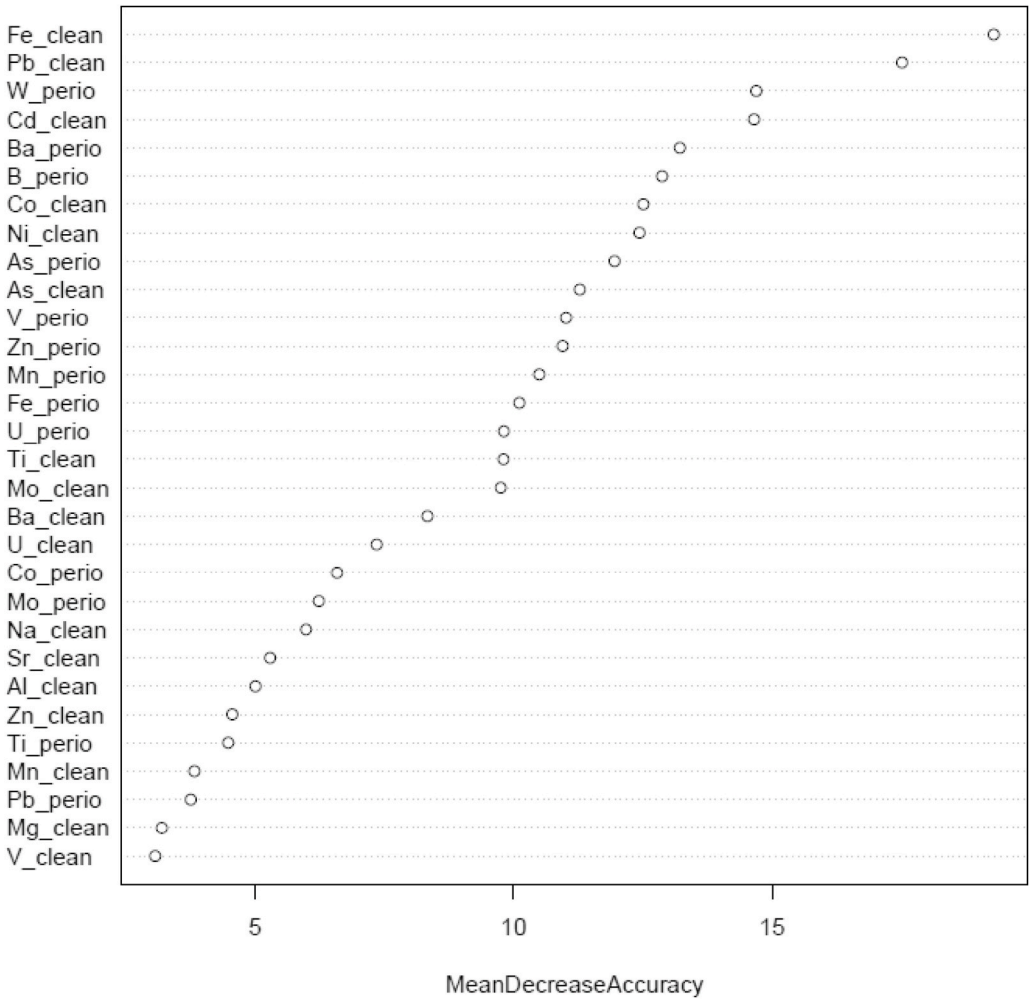

Fig. 3. Variable importance of all variables from the random forest analysis with the highest rate of classification success of mussels to their date of harvest, based on the elemental composition of the clean shell and periostracum, according to the mean decrease in accuracy measure.

perhaps unsurprising that classification success decreased somewhat over time (Table 2 & Fig. 2), as it is expected that reference chemical signatures would fluctuate overtime, as has been illustrated in the past by biomonitoring and bivalve larvae tracking studies (Becker et al., 2005; Bellotto and Miekely, 2007; Dunphy et al., 2011).

Consistently the models were able to successfully classify all individuals correctly to January 2016. Primarily, this could be attributed to the length of time between the next subsequent sampling event (January 2017). This gap between sampling occasions of one year provided a higher likelihood of retention of different concentrations of reference chemical signatures within the soft tissues and in particular, the shells. It is well understood that the bioavailability of trace elements in the environment varies seasonally (Rainbow et al., 2004) attributed to seasonal factors such as precipitation and temperature influencing biogeochemical processes. Though it is possible that seasonal patterns exist too, on several occasions, the model had difficulty distinguishing between Jan 2017 and Jan 2018 (Table 2, periostracum). This could be due to seasonal patterns of bioavailable elements. As this was a bigger issue for soft tissues as opposed to shells, it is likely this is due to seasonal patterns as these would be more readily incorporated in soft tissues (due to turnover rates) compared to the shells.

Iron, lead and to a lesser extent, tungsten and cadmium, were the most important elements in correctly identifying the harvest date of individuals as indicated by the random forest analysis. The soil type in the catchment around the study site consists primarily of peat soils and runoff from such soils is particularly rich in dissolved humic-bound iron, the concentration of which and the export to marine waters, is highly dependent on seasonal changes in rainfall (Krachler et al., 2010; 2016). Similarly, the concentrations of lead are associated with the dissolved or colloidal organic matter levels in seawater, which varies seasonally (Wallace, 1982). Tungsten concentrations in seawater displays complex relationship with seasonal differences in salinity (Mohajerin et al., 2016). Several studies have highlighted seasonal variations in the concentrations of metals and other elements in *Mytilus* species including iron, lead, cadmium and tungsten, due to various factors including seasonal bioavailability in seawater, reproductive state of the bivalves and temperature (Azizi et al., 2018; Rainbow et al., 2004; Richir & Gobert, 2014; Rouane-Hacene et al., 2015) as well as site specific factors (Bennion et al., 2019; Morrison et al., 2019). The abiotic and biotic factors that influence the bioavailable concentrations of these elements and others in seawater and are hence incidentally incorporated into both the shell and soft tissues of these animals, are therefore complex (Bennion et al., 2019; Morrison et al., 2019). However, as many of these factors impact elements in different manners both temporally and spatially, this complexity may facilitate unique TEF of bivalves that can be used to identify both the time as well as the location of harvesting.

The potential of mass-spectrometry, TEF-based traceability tools has been shown, albeit with varying success, for cockles *Cerastoderma edule* (Ricardo et al., 2015a, 2017a) and goose barnacles, *Pollicipes pollicipes* (Albuquerque et al., 2016) in Portugal, green-lipped mussels, *Perna canaliculus* in New Zealand (Dunphy et al., 2015) and manila clam, *Ruditapes philippinarum* in China (Zhao et al., 2019). More recently

again, using a combination of laboratory and field-based experiments Honig et al. (2020) showed the viability of shell TEF as a provencing tool for *M. edulis* in the Gulf of Maine, USA for conservation purposes. However, the use of TEF based on the elemental concentration of both the shell and the soft tissues has shown by far the greatest success rates, of 100% of individuals blue mussels, *M. edulis* (Bennion et al., 2019) and king scallops, *Pecten maximus* (Morrison et al., 2019) to their site of harvest in Ireland. Soft tissues uptake bioavailable elements at different rates and concentrations than calcium carbonate structures such as shells (Bellotto & Miekeley, 2007; Szefer et al., 2002). The rapid turnover rate of soft tissues compared to calcite structures impacts their reliability and thus their use within traceability studies. For example, shells are better for use in biomonitoring studies that examine bioavailability of trace elements over a longer temporal scale compared to soft tissues due to the more rapid turnover of soft tissues. This has consequences for traceability, as the stability of elemental signatures within shells compared to soft tissues makes them more reliable over time, though this reliability reduces the potential for small scale changes in bioavailable elements to be identified during analyses. For this reason, the shells provide a reliable base for analysis to distinguish samples between sites (where variation of bioavailable elements is likely larger). The soft tissues have a rapid turnover, making it more likely that incremental changes in elemental bioavailability will be picked up by analyses. This means that soft tissue micro-chemistry is more crucial when attempting to distinguish between sites within the same estuary or harbour, or within a smaller temporal scale (month to month).

Ricardo and co-authors (Ricardo, Pimentel, Génio, & Calado, 2017) noted that the most fundamental restriction to TEF-based tools is the need to regularly update the reference chemical libraries. Morrison et al. (2019) subsequently demonstrated that the TEF based on the shell and soft tissue of king scallops varied considerably over even relatively short periods. Therefore, the logical next step, to test the protocol described here, in Bennion et al. (2019) and Morrison et al. (2019), was to assess the temporal stability of TEFs and thus infer the sampling regularity needed to update the reference chemical library. The fact that TEF based on shell and soft tissue are so distinct between sampling events as demonstrated both here with blue mussels and with king scallops by Morrison et al. (2019) is certainly beneficial as it allows not only the identification of harvest site but also the time frame. A well-maintained reference chemical library is therefore the key to the success of a TEF-based tool, to ensure correct classification over time and between spatially disparate locations. As Milan et al. (2019) highlight for next-generation DNA sequencing (NGS) based tools, this need for regular sampling to maintain any reference library could provide an opportunity to monitor and conduct research simultaneously. Bivalves and other molluscs have been and continue to be instrumental as biomonitors as evidenced by long-term data sets such as, The MusselWatch programme (Goldberg, 1986). A carefully designed sampling strategy, to maintain critical databases necessary and facilitate classification success rates spatio-temporally, could provide a substantial resource in the form of systematic and continuous, parasite, pathogen and trace metal pollution monitoring.

Fortunately, the incidence of counterfeit molluscan produce making it to market, and illness arising from the consumption of contaminated shellfish, are a relatively rare occurrence. In the European Union in 2017, 82 outbreaks were attributed to fish products and 24 were attributed to crustacean and molluscan products combined (EFSA, 2018). This is largely thanks to the efforts of industry regulators who uphold food safety legislation in place. That being said, the consequences for the marketplace, producers and consumers are still great, as recent studies into seafood fraud have revealed (Fox et al., 2018; Warner et al., 2013) such as the mislabelling of sushi products (Warner et al., 2013) and of course, beluga (*Huso huso*) caviar (Ludwig et al., 2015). A vast number of livelihoods in job poor-regions are supported by molluscan aquaculture (FAO, 2018). Producers are already intermittently tested by the occurrence of mass mortality events (Soon & Ransangan, 2019), the mitigation of which are extremely challenging as deciphering their cause is a complex and time-consuming undertaking. The risk to these job-poor regions is therefore significant and requires a bigger safety-net. Lately, a shift in consumer perceptions has occurred and a greater value has been placed in food traceability (Van Rijswijk & Frewer Lynn, 2008). This is evidenced by the growing number of traceability-focused studies (referenced herein) and food and ingredient traceability-specific legislature being established (e.g. General Food Law, Regulation (EC) 178/2002 in the European Union). Initiatives such as 'Farm-to-fork' (Dowling et al., 2009) show the rapid movement in this area for terrestrially sourced products however, the establishment of similar tools for molluscan produce has been much slower, likely attributed to the semi-controlled nature of bivalve aquaculture, versus the tightly controlled and more easily managed land-based farms. Though initiatives like 'FishPopTrace' show a recognition of this need within the aquaculture industry too (https://fishpoptrace.jrc.ec.europa.eu/).

To that end, a standardised scientific method for tracing molluscan produce to its source is needed to accelerate tractability protocols and give power to regulators to overcome the rising tide of food fraud and counterfeit produce. Bennion et al. (2019) showed how the use of a combination of structures from blue mussels, in particular the most recent growth annuli of shells, allowed 100% classification success of mussels to rearing location, overcoming the issues associated with the buying and selling of spat such as TEFs prior to settlement frustrating the TEF for respective rearing sites or the limitations of genetic based approaches (Bennion et al., 2019). Following on, Morrison et al. (2019) showed how the same method could be applied to king scallops, to predict harvesting location but also harvesting period (six weeks apart), again with 100% classification success. Here, the temporal variability of TEF has been shown, as 100% classification success could not be achieved between January 2016 and 2018. Based on the combination of the trace element signatures of the clean shell and the periostracum combined, the model was able to predict harvesting period between in January 2016, January 2017 and May 2017 (Table 2 & Fig. 2). Importantly, the model could not provide 100% classification success for September 2017 and January 2018, misclassifying one individual from September 2017 to May 2017 and January 2018 to September 2017. This result illustrates the need to update reference chemical libraries regularly to ensure correct classification success over time. This recommendation mirrors the findings of Ricardo and co-authors (2017a), where results indicated a need to update reference chemical libraries after six months and before one year.

5. Conclusion

Molluscan bivalve producers and industry regulators will continually be challenged by both HABs and food fraud. As global consumption of seafood rises, IUU and the presence of inferior and counterfeit produce within the marketplace is likely to increase also. Traceability tools offer a failsafe for regulators to combat such fraud and minimise the potential negative impact HAB could have on human health. Consumers are becoming increasingly aware of the problem of food fraud and their concerns warrant action to restore trust, protecting producers, workers and the industry as a whole. TEF-based traceability tools that analyse both the shell and soft tissues of animals have been proved a good fit for tracing bivalve molluscan produce to its source (over time and space). Reference libraries will need to be continually maintained to ensure predictive success over time.

This scientifically based traceability tool has been shown to work across taxa (Bennion et al., 2019; Morrison et al., 2019), going forward, efforts relating to these TEF tools should be intensified. An exploration of other commercially important taxa such as oysters and indeed crustacean species in addition to a fine scale examination of the temporal and spatial resolution possible is critical next step to test the rigour of this method.

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Declaration of competing interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

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