DOCTOR OF PHILOSOPHY

The use of biogeochemical tags to determine the origins and movement patterns of fishes

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Award date:
2014

Awarding institution:
Bangor University

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A thesis presented for the Degree of Doctor of Philosophy at
Bangor University

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November 2013

School of Ocean Sciences

Bangor University

Funding for the research was provided by the Natural Environment Research Council (NERC) and the Centre for Environment, Fisheries and Aquaculture Science (Cefas: CASE Partner)

Abstract

The incorporation of both trace and minor-trace elements within the otolith aragonite matrix of hatchery reared sea bass *Dicentrarchus labrax* and the possible effects of *post mortem* handling, transportation and the period of time whole fish were stored frozen were examined. Furthermore, the possible effects of temporal variability of the water chemistry within two nursery grounds and the effect on the elemental concentrations within otoliths were measured. Finally, the use of naturally occurring trace and minor-trace elements incorporated within the otolith structures of teleost fish and their use as natural biogeochemical tags to infer movement patterns over spatial scales were assessed.

Statistically significant differences were observed in the concentrations of Mg and K measured in the otoliths of hatchery reared *Dicentrarchus labrax* when whole fish were stored frozen for a period of 6 months. Similarly, the elemental concentrations of Mn differed significantly between the storage periods of 1 day and 12 months. Three elements Na, Sr and Ba indicated no significant change in their elemental concentrations in response to the methods of dispatch, transportation protocol and freezer storage period. Based on the concentrations of Na, Sr and Ba, indications show these three elements are not subject to alteration when using the most commonly used methods of euthanasia / transportation and storage duration for *D. labrax*.

Significant inter-annual and intra-annual differences were observed in the elemental concentrations of otoliths from juvenile *Pleuronectes platessa* sampled over a period of 7 years (2004-2010) from two nursery grounds Llanfairfechan and Llandonna in North Wales. Inter-annual (between years) variation at the site Llanfairfechan was observed for Mg in each of the 3 years 2007-2010, similarly between the 2 years 2009-2010 and the concentrations of Na. Differences were also observed in the concentrations of Sr and between each of the 5 years 2005-2010. Inter-annual variation was observed at the site Llanfairfechan for Na between each of the 3 years 2007-2010, between the 2 years 2007-2010 for K, and between the two years 2007-2009 and 2009-2010 for Sr and Ba respectively. Some degree of temporal stability could be observed for Na, Mg and Ba at Llanfairfechan and for Na, K, Sr and Ba within the site at Llandonna over short time scales (*i.e.* 2-3 year periods), increasing to 4 years (2004-2007) for Na and Ba at Llanfairfechan and K and Ba at Llanddona. There appeared to be some temporal stability on an inter-

annual scale over a short term: *i.e.* 2-3 years, with some elements such as Ba being more stable for a period up to 4 years.

Significant differences in the elemental concentrations of Mg, Mn, Sr and Ba were observed in the otoliths of *Salmo trutta* parr sampled from each of 36 main sea trout producing rivers in SW Scotland, NW England, Wales, Isle of Man and the east coast of Ireland which flow into the Irish and Celtic Seas. Using quadratic discriminant function analysis (QDFA), 74% of juvenile trout parr were classified back to their natal rivers, with 66% of trout parr correctly classified to region. Using the elements Mg, Mn, Sr and Ba from trout parr otoliths a freshwater biogeochemical baseline was produced to assign "blind" run parr samples to their source, with 27/39 (69%) of "blind" run trout parr correctly classified back to their natal river. Using the biogeochemical freshwater baseline created from the QDFA adult sea trout of unknown origin were assigned to their putative natal region using their period of freshwater residency. Classification was low with > 20% of adults correctly assigned to their putative region of origin. Although classification accuracy of trout parr to river / region was high and indicated the freshwater baseline was robust, the results for the present study suggest that the poor classification of adults to their putative natal region may indicate migratory patterns for adult sea trout within the Irish Sea are more extensive than previously understood.

Acknowledgements

To even begin to thank all of those who in some part were involved or indeed assisted with the creation of this thesis would be an impossible task. So I will thank all of those who I may have forgotten over the last 4 years in advance. Thank you.

Firstly, I would like to thank my project supervisors Dr. I. D. McCarthy and Prof. C. A. Richardson (S.O.S. Bangor University), Dr. S. R. N. Chenery (B.G.S. Nottingham) and Dr M. Armstrong (CEFAS; Case partner) for all their encouragement and helpful advice on the project during the long meetings over copious amounts of coffee, tea and cake. I would also like to thank the Centre for Environment, Fisheries and Aquaculture Science (Cefas) and The Natural Environment Research Council (NERC) who kindly funded this research project.

I would particularly like to thank Ian for his helpful comments on the early stages of the project and on earlier drafts of my thesis and his support and direction when the initial ideas for the project failed to materialise. I would also like to say a special thank you to Simon, who took time out of his busy schedule to explain the fundamentals of microchemistry, the use of the LA and sb-ICP-MS and how to interpret the results over the long weekends we had at the B.G.S. Thanks for the coffee.

I would also like to thank the team at the Environment Agency (EA), Walli Hanks, Pierino Algieri, Richard Prichard, Richard Pierce and Gethyn Morris in assisting in the attempt to collect sea bass for the failed early part of my thesis. I would also like to thank The Celtic Sea trout Project (C.S.T.P.) who, with the direction and supervision of Ian McCarthy allowed me to take on part of the microchemistry work as a filler for my research project when all seemed lost. Thanks must also go to the Ireland Wales Territorial Co-operation Programme 2007-2013 (INTERREG 4A) for funding the C.S.T.P. project. My thanks also go to Dr Clive Truman and Dr Matt Cooper at the National Oceanographic Centre, Southampton (N.O.C.S) for their help and advice during sample analysis at the N.O.C.S.

I wish to also like to thank all the technical staff here at the School of Ocean Sciences and especially Joan Griffiths, Lynne Roberts, Gwyn Hughes, Ian Prichard, Gwynne Jones and Vallan Astley for making me feel at home and smile when all seemed lost, Diolch yn fawr. Special thanks must go to "my partner in crime" Berwyn Roberts for assisting me in the

long days and even longer nights in sample collection around the cold coastal waters of North Wales. Dry suits should not be worn as an alternative to a floatation suite "This way up!"

A special thank you must go out to all of my friends, colleagues and staff here at the School of Ocean Sciences. I would also like to thank Alice Ramsay for her invaluable assistance in the early parts of my research and of course John Latchford and Cara Hughes for assistance in the queries I had regarding statistics Beers on Me! I would especially like to thank Michael Watts for allowing me to reside for long hours and weekends in the office and the laboratory at the B.G.S. while I was running my samples. I would also like to express my thanks to the staff and my work colleagues at the B.G.S. for their invaluable help and for making me feel at home and part of the team.

Thanks must also go to all my friends here in Menai Bridge who over the last 4 years made me feel welcome and at home: Maldwyn Jones and Lynne Roberts for trying to get me to go out when I was lost in my own little world. Mark Prest both Hefin Rowlands and Hefin Jones, Little Rita, Mark Rees, Pete Warder, Ian Evans for making evenings out very entertaining and to the rest of the Bulkeley Arms "cheers". I would also like to say a special thank you of course to my long suffering house mates (past and present) but especially to Adam Fetherstonhaugh, Claire Catherall, Vicki Greenhalgh, Claudia Tanneberger and Lauren D'Alessandro-Heath who ALL took time out to listen to my endless conversations about *Fisheries Science* and kept me sane during the long hours I locked myself away. I would also like to extend my thanks to my family and my friends back home in the Midlands who never doubted my ability even when I did.

Last, but by no means least, I would like to thank Ruth Jones. You are one of the most unselfish people I have ever had the pleasure of knowing. Your 'You can do it' attitude and endless encouragement when my spirits were low throughout my 4 years of study here at Bangor were inspiring. A true friend and Soul Mate. Thank You.

To my Mother

You were always by my side in mind and now in spirit
You always believed in me no matter what direction I took
I have now finally achieved my dream
I wish you were here to see it
Thank You

Thesis Contents

Chapter 1

General Introduction

1.1 Migratory patterns:

Where do they originate from and where do they go too?

It is well known that some fish species undertake complicated and varied movement patterns between very distinct water bodies during their life cycles (Cadrin *et al.*, 2013), with movements confined to wholly freshwater or marine environments or transitional between the two when studying diadromous species. For fisheries management, conservation and exploitation purposes it is fundamental that we understand their ecology and movement patterns during their life time if we are to utilize them sustainably. Freshwater species such as resident brown trout *Salmo trutta* can undertake short migrations of a few kilometers within streams for feeding or to find areas suitable for spawning (*e.g.* Vøllestad *et al.*, 2012). In contrast species such as the paddlefish *Polyodon spathula* are known to be highly mobile making extensive movements (*ca.* 2,000 km) within river systems and are frequently capable of covering large distances (*ca.* 40 km) within a 24 hour period (Jennings and Zigler, 2000). One of the longest freshwater migrations observed is the Amazonian catfish *Brachyplatystoma rousseauxii*. Migrations of these species regularly exceed 10,000 km between juvenile nursery areas in the estuary of the Amazon to breeding zones in the head waters of the western Amazon basin close to the Andes (Garcia Vasquez *et al.*, 2009).

Marine species too have variable migratory patterns, with large-scale movements in the order of 100s of km between nursery, feeding and spawning grounds not uncommon. North East Atlantic examples of these include Atlantic herring *Clupea harengus* (Ruzzante *et al.*, 2006), European plaice *Pleuronectes platessa* (Dunn and Pawson, 2002) and European sea bass *Dicentrarchus labrax* (Pawson *et al.*, 2007). Some fishes however, undertake transoceanic migrations covering several 1000 km's, such as the feeding migrations of salmonids while at sea (Groot and Margolis, 1991; Hansen and Quinn, 1998). Greater migrations can be observed for the European eel *Anguilla anguilla* which undertakes migrations of *ca.* 5,000 km from its feeding grounds in European freshwaters to spawning grounds in the Sargasso Sea (Aarestrup *et al.*, 2009). The marine species which undertakes the longest transoceanic migration is probably the white shark *Carcharodon carcharias*, with one individual recorded as undertaking a migration from South Africa to Australia and back, a journey in excess of 20,000 km (Bonfil *et al.*, 2005). Evidently, both marine and freshwater fishes undertake movements of

considerable magnitude during differing stages of their life cycles. The main questions facing both managers and scientists alike are where have those fish come from? (*i.e.* natal origin) and more importantly where and why do they go? (*i.e.* movement patterns).

1.2 Tracking migratory patterns:

Can we determine these movement patterns?

The terrestrial movement patterns of animals have been easier to understand and track due to their coexistence within the same environment as man. However, this is not as easily undertaken when trying to observe the movement patterns of aquatic animals. By the nature of the medium in which they live – water, compounded by the vast three dimensional environments of the oceans, we as humans cannot easily observe where they go to and where they come from. Despite these difficulties, attempts have been made to track movement patterns and identify origins of fish as early as the 1880s using external tags attached to the fish (Loerke & Cadrin, 2007). There are two methods readily used in trying to track movement patterns of fish, the use of "applied" markers where artificial tags are introduced to the fish by researchers and "natural" markers where unique natural characteristics are used in identifying origins of the individual fish and used to observe movement patterns (see Cadrin *et al.*, 2013 for a detailed review of the subject area).

Applied tags can be either placed on the external surface of the aquatic animal allowing the tag to be visually identified if recaptured or implanted within the body (*i.e.* PIT tags in fish) and vary dependent upon species, time and more importantly funding, with some types of tags costing nothing *e.g.* notching / fin clipping, up to a few thousand pounds each *e.g.* satellite tags. For aquatic animals tags include: V-notching (in crustaceans) or fin clipping (in fishes), polyethylene or rubber ribbons / discs (in shellfish), visible implant elastomer (VIE, in fishes), anchor tags (in fishes), laminated disc tags (in flatfishes), passive integrated transponders (PIT tags, in fishes), acoustic tags (in fishes; used in radio-tracking), archival data storage tags (DSTs, in fishes) and "pop up" satellite tags (in fishes) (see Cadrin *et al.*, 2013 for a detailed review of the range of applied and natural markers used in fish biology).

The use of conventional tag-recapture and radio-tagging of individual fish has assisted in reconstructing the movement patterns of various species (see reviews by

Cooke *et al.*, 2011 and Cadrin *et al.*, 2013). These methods are conducted frequently by Government fisheries agencies monitoring fish stocks and management programmes *e.g.* large-scale tagging programmes in the UK on European plaice (Dunn and Pawson, 2002) and European sea bass (Pawson *et al.*, 2007) by Cefas to inform ICES on management policies. However, these tagging programmes are extremely labour intensive and logistically difficult to implement (tag and recapture rates can be very low see King, 2007; *e.g.* see Herzka *et al.*, 2009). Furthermore, cost can be high depending upon the number of samples to be targeted and the equipment used *(i.e.* radio tags, monitoring equipment), with financial constraints reducing the number of fish to be monitored and other costs incurred due to lost or damaged equipment (*e.g.* loss of expensive radio satellite tags), combined with poor returns from recaptured or successfully tracked fish (Cadrin *et al.*, 2013). More so, we must consider the gains in our knowledge from using successful tagging programmes when comparing costs incurred for time and equipment (*e.g.* Block *et al.*, 2001; Bonfil *et al.*, 2005; Galuardi and Lutcavage, 2012). Methods using tags such as these have increased our understanding of homing traits observed for anadromous adults to their natal rivers which have proved difficult in the past using many conventional tagging methods.

Some success can be attributable to more advanced technology using archival and satellite radio tags (Dingle, 1996; Elsdon *et al.*, 2008). Miniaturized artificial radio tags combined with more sophisticated radio telemetry have enabled "tiny" PIT tags to be developed that have been used to document behaviour in ants (Moreau *et al.*, 2011) and bees (Decourtye *et al.*, 2011), with tags of *ca.* 6mm in length used on studies of small zebra fish (size range 16-42 mm; Cousin *et al.*, 2012). However, cost and the use of many conventional external tags have hindered our understanding of natal origins and early movement patterns of juvenile fishes due to size limitation *i.e.* high mortality observed for small fish during the tagging process (see review in Cadrin *et al.*, 2013). This problematic subject is therefore only feasible when the fish have attained a size at which the impact of attaching / implanting the tag will no longer affect their survival or growth (*e.g.* Ombredane *et al.*, 1998; Richard *et al.*, 2013). However, such an understanding of the early life history patterns is fundamental if we are to further understand their population structure and movement dynamics (Kennedy *et al.*, 2002; Metcalfe *et al.*, 2002).

The understanding of movement patterns and life history strategies is not only confined to juvenile fish. Tracking and monitoring of adults within the marine

environment too can have its problems using traditional tagging techniques. The number of fish required to be tagged and the time required to get meaningful results to elucidate stock structure and movement patterns can be immense (*e.g.* 30+ years monitoring programme on the European sea bass in UK waters. Holden and Williams, 1974; Kelley, 1979; Pawson *et al.*, 1987; Pawson *et al.*, 2007; Pawson *et al.*, 2008; Quayle *et al.*, 2009).

1.3. Natural markers

An alternative approach to the sometimes costly and time consuming use of artificial tagging in both adult and juvenile fish in helping to understand geographical distributions, dispersal and movement patterns has been the use of natural markers (Walther and Thorrold, 2009; ICES, 2012). Natural markers come from a variety of applications, and can be used to look at movement patterns and help in identifying origins of an individual animal using one or more of their unique natural characteristics. These natural markers have included the use of parasites, bacterial communities, distinctive body markings, meristics / morphometrics, genetics and the use of chemical tags such as stable isotopes and trace element microchemistry.

Parasites have been used as a tool for stock discrimination of both demersal and pelagic marine fish species as well as anadromous salmonids, cetaceans and invertebrate species (see Mackenzie and Abaunza, 1998; Mackenzie, 2002). Their use as biological markers in identifying origin or movement has also been studied. Mackenzie and Abaunza (1998) and Mackenzie (2002) showed where parasitic infection had been found to be endemic to a specific geographical region; animals which were subsequently caught outside that region but were infested with these site-specific parasites had at some point visited that area during part of their life history. Similar studies using bacterial populations which are associated with the mucus layer of fish and surrounding seawater have been used to infer origins in gadids (Wilson *et al.*, 2008; Smith *et al.*, 2009) and on a more global scale in tracing origins of marine ornamental fishes (Cohen *et al.*, 2013).

Another method used in the identification of individuals in some aquatic species and to track their movement is the use of natural body markings such as tears, marks, notches and scars in fins and tail flukes, and spot patterns. Mainly adopted for the use with marine megafauna *e.g.* large elasmobranchs (Castro and Rosa, 2005; Van Tienhoven *et al.*, 2007) and cetaceans (Dufault and Whitehead, 1995), this approach has also been

used in identifying small juveniles in some species like salmonids (Leaniz *et al.*, 1994; Donaghy *et al.*, 2005; Merz *et al.*, 2012).

One extensive method used in understanding population structure is the use of genetic markers, with these markers used for population differentiation (Hamilton, 2009; Nielsen and Slatkin, 2013), stock discrimination for fisheries management purposes (Carvalho and Hauser, 1994; Shaklee *et al.*, 1999) and to identify and conserve species of high genetic pedigrees (Avise, 1989; Hedrick, 2001). Changes in how genetic studies were conducted have enabled a more precise understanding of stock structures, with studies using protein polymorphism giving way to the use of molecular DNA polymorphisms using microsatellites and mitochondrial DNA (Begg and Waldman, 1999; Okumuş and Ciftci, 2003). However, the standard tool for the use in assessing population variation in fishes for the use of exploitation and conservation is the use of nuclear DNA (*e.g.* Sato *et al.*, 2004). One group of fish whose population structure and genetic diversity has been extensively studied are the salmonids (*e.g.* Sato *et al.*, 2004; Verspoor *et al.*, 2007). Studies using genetics have looked at both large geographical and spatial scales (*e.g.* Utter *et al.*, 1989; Bernatchez *et al.*, 1992; King *et al.*, 2001; Sato *et al.*, 2004) to the smaller fine-scale populations observed within a single river catchment (*e.g.* Carlsson and Nilsson, 2000; Kitanishi *et al.*, 2009; Stelkens *et al.*, 2012), with very good results.

1.4. Biogeochemical markers

The use of natural biogeochemical markers in animal tissues and hard parts (*e.g.* scales, fin rays and otoliths) have provided research scientists with alternative marking methods for species which have been previously difficult to tag using conventional techniques (Walther *et al.*, 2008). One method growing in interest to study the origins and movement patterns of animals is the use of stable isotopes (Rubenstein and Hobson, 2004: Graham *et al.*, 2010; Hobson *et al.*, 2010; Trueman *et al.*, 2012; McMahon *et al.*, 2013). Using differences observed from the isotopic composition of water (West *et al.*, 2010) isotopes such as nitrogen ($\delta^{15}N$), carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$), sulphur ($\delta^{34}S$) and strontium ($\delta^{87}Sr$) have been used to infer origins and movement patterns of fish within freshwater, marine and estuarine environments (see Kennedy *et al.*, 2005; Barnett-Johnson *et al.*, 2008; Zeigler and Whitledge, 2010; Trueman *et al.*, 2012; Martin *et al.*, 2013; McMahon

et al., 2013). Most studies of this nature have tended to examine large scale movement patterns within the marine environment (*e.g.* bluefin tuna *Thunnus thynnus*, yellowfin tuna *Thunnus albacares* and swordfish *Xiphias gladius*; Ménard *et al.*, 2007; Rooker *et al.*, 2008; Graham *et al.*, 2010). However, interest in studying the movement patterns of fish within the freshwater environment through the application of these isotope markers is growing (*e.g.* Kennedy *et al.*, 2005; Barnett-Johnson *et al.*, 2008; Zeigler and Whitledge, 2011; Martin *et al.*, 2013). One such application using brown trout *S. trutta* scales and the isotopes $\delta^{13}C$ and $\delta^{15}N$ enabled brown trout to be classified back to their site of origin within the River Dee catchment in North Wales with a high degree of accuracy (Ramsay *et al.*, 2012).

Since the 1970's interest in the use of calcified structures and their chemical composition to address wide ranging questions in fisheries science has grown. This concept assumes the chemical composition of these structures reflects broadly the physio-chemical characteristics of the environment to which those fish were exposed (de Pontual and Geffen, 2002). One such application proving to be an extremely valuable tool for studying movement and life history characteristics of teleost fishes is the use of microchemistry, more specifically the use of calcareous structures such as fin rays, vertebrae, fin scales and otoliths (*e.g.* Wells *et al.*, 2003*b* Clarke *et al.*, 2007; Elsdon *et al.*, 2008; Ramsay *et al.*, 2011; Tillett *et al.*, 2012). One of the more commonly used calcified structures in microchemistry studies, fish otoliths, have been often referred to as a continuous recorder of environmental exposure (Campana *et al.*, 1997), as their composition has one of the highest potentials to convey environmental information compared to other calcified structures (de Pontual and Geffen, 2002; Elsdon *et al.*, 2008).

Otoliths (or ear stones) are paired calcareous structures within the inner ear of fish that have been routinely used as biochronological markers for aging of fish due to the daily cyclic deposition of crystalline (*i.e.* calcium carbonate) and organic material (*e.g.* proteins: Campana and Neilson, 1985; Elsdon *et al*,. 2008) forming concentric rings over the life time of the fish (Beamish and McFarlane, 1987). However, due to the metabolically inert nature of these calcium carbonate structures (more specifically in its aragonite morph: see Degens *et al.*, 1969; Mugiya, 1972; Radtke, 1978), the mineralogy of the otoliths is thought to remain unaltered after the sequential deposition of the calcium carbonate. Composed of 99% $CaCO_3$ the metabolically inert nature of otoliths, combined with trace and ultra-trace elements which make up the remaining 1% (see Campana and Neilson, 1985; Payan *et al.*, 2004) are derived in some degree from the

elemental chemistry of the surrounding ambient water (Farrell and Campana, 1996; Bath *et al.*, 2000; Walther and Thorrold, 2006; Jolivet *et al.*, 2013). Generally considered as biological archives (Campana, 1999; Thresher, 1999; Payan *et al.*, 2004; Jolivet *et al.*, 2008), these unique chemical properties allow otoliths to be used as natural biogeochemical markers (Campana, 1999).

1.5. Otolith microchemistry

To understand the incorporation of trace elements into the otolith, the original chemical composition of the structure and its ability to act as a natural elemental tag in fish population studies should be described. Fish otoliths are paired structures located within the inner ear of all bony fish and used for maintaining equilibrium, processing directional cues and sound reception (Wright *et al.*, 2002; Popper and Fay, 2011; Sturrock *et al.*, 2012). Comprised mainly of calcium carbonate ($CaCO_3$) aragonite crystals crystallized onto a fibroprotein organic matrix (Dannevig, 1956; Degens *et al.*, 1969; Campana & Neilson, 1985; Wright *et al.*, 2002; Tohse & Mugiya, 2002; Payan *et al.*, 2004), otoliths grow continuously throughout the life history of the fish from the early embryonic stages to the time of its death (Campana & Neilson, 1985; de Pontual and Geffen, 2002; Elsdon *et al.*, 2008). Studies conducted on a number of broad elemental assays have shown that otoliths are relatively pure, with total inorganic impurities comprising less than 1% of the total otolith weight (Edmonds *et al.*, 1992; Thresher *et al.*, 1994; Proctor *et al.*, 1995; Campana *et al.*, 1997). A comprehensive review by Campana (1999) indicated a total of 31 major (Ca, C, O and N), minor (> 100 mg kg^{-1}) and trace (< 100 mg kg^{-1}) elements have been detected in otoliths, with otolith elemental composition mainly dominated by calcium (Ca), oxygen (O) and carbon (C), with other elements such as Na, Sr, K, S, N, Cl and P present at concentrations > 100 ppm, and the so-called "trace" elements at concentrations < 100 ppm. However, with the improvement in analytical detection limits during the last decade the number of detectable elements in larval and adult fish otoliths has increased to 50 (Chen and Jones, 2006; Sturrock *et al.*, 2012).

Analytical techniques used to assess the elemental composition of fish otoliths require the $CaCO_3$ polymorph to be in its normal crystal morph which for the sagittae and lapilli otoliths is in the form of aragonite. However in some circumstances this morph can be replaced (often only partially) by vaterite (or more rarely calcite) to produce what are

known as "crystalline" otoliths (Gauldie *et al.*, 1993; Wright *et al.*, 2002) which differ in their chemical uptake of the minor and trace elements. de Pontual (unpublished data, cited in Wright *et al.*, 2002) showed decreases in concentrations of Sr, Na and K in the vaterite portion of the otolith, with Mg and Ca showing higher concentrations than observed within the normal aragonite otolith. Brown and Severin (1999) also reported a decrease in concentrations of Sr, Na and K within vaterite otoliths, but did not report any findings on Mg concentrations. Studies have shown that the otolith is impregnated with the endolymph (a liquid with special viscous properties; Wright *et al.*, 2002) in which it bathes (Gauldie and Coote, 1997; Gauldie and Cremer, 1998; Milton and Chenery, 1998; Proctor and Thresher, 1998). Furthermore, Thresher (1999) has commented that the fluid component of the endolymph is a major influence in determining the ultimate composition of the otolith as the calcium carbonate precipitates out from the endolymphatic fluid (Miller *et al.*, 2006). Wright *et al.*, (2002) found that the principal components of the endolymph display decreasing or increasing proximodistal concentration gradients that may act as a driving force in the biomineralization processes, enabling heterogeneous distribution of some elements on the otolith surface.

The endolymph proteins have also been recognised as playing a pivotal role in the uptake and inhibition of calcium carbonate as well as other processes of biomineralization and organomineralization (Trichet and Defarge, 1995). Campana (1999) has described how the calcification process of the otolith is heavily dependent upon the composition of the endolymphatic fluid surrounding it and concludes that one of the key physical regulating factors of the endolymph is pH, which is determined by the concentration of bicarbonate ions within the endolymph (bicarbonate is one of the ion products of carbon dioxide in solution; Romanek and Gauldie, 1996; Payan *et al.*, 1997). Alkalinity within the endolymph is regulated by proton secretion through the saccular epithelium, which reduces the rate of calcification (Payan *et al.*, 1997).

However, few studies have been conducted on the endolymph fluid and the organic components of the otolith, and additional studies are therefore critical if we are to fully understand the processes of otolith formation and composition (Wright *et al.*, 2002). However, the presence of a pH gradient within the inner ear of teleost fish and its relationship to biocalcification rates (see Gauldie and Nelson, 1990; Gauldie *et al.*, 1995; Payan *et al.*, 1997) through a pH-dependent proton secretory mechanism, may adversely affect the patterns of elemental deposition through changes in endolymph pH during periods of trauma (*e.g.* stress: affecting blood pH and in turn affecting endolymph pH).

1.5.1 Otolith elemental assimilation

The processes involved in otolith formation are unlike those of biomineralization of molluscan shells and coral skeletons in that the epithelium of the otolith is not in direct contact with the region of calcification or with the surrounding water. Indeed, the process of calcification can be described to a certain extent as being based on purely physical principles being acellular and heavily reliant upon the composition of the endolymphatic fluid that surrounds and bathes the otolith (Campana, 1999; Wright *et al.*, 2002; Payan *et al.*, 2004). The incorporation of environmental factors into the composition of the otolith is a complex process which according to Wright *et al.*, (2002) involves four nested compartments: (1) the external medium, where variations in abiotic factors occur; (2) the blood plasma, which is respondent to the external medium and also exhibits endogenous variation; (3) the endolymph, which modulates the various signals and regulates the formation of the otolith; and (4) the otolith, which integrates and records responses to all these signals.

The basic pathway for the uptake of inorganic elements into the otolith is from the surrounding medium (water) into the blood plasma via the gills or intestine. These are then transported into the endolymph and finally precipitated into the crystallizing otolith (Campana, 1999; Wright *et al.*, 2002, Miller *et al.*, 2006). In freshwater fish, branchial uptake (via the passing of water over the gills) is the primary source of elements, while the main source of water-borne elements for marine fish is through the continual action of drinking water, which is then absorbed through the intestinal epithelium as part of their osmoregulatory process (Simkiss, 1974; Olsson *et al.*, 1998; Wright *et al.*, 2002). There is, however, a small, unknown fraction of elements that can be incorporated into the blood plasma via the fish's diet through the digestion of food (*e.g.* Sr), (Farrell and Campana, 1996; Gallahar and Kingsford, 1996). However, the rate of assimilation of minor elements into the otolith in this manner is considered minimal according to a study by Hoff and Fuiman, (1995).

1.6. Otolith microchemistry: Metabolically inert

It has been assumed that the otolith is metabolically inert and acellular in nature and that its overall composition is not subject to dissolution, reabsorption or erosion, however, this

assumption has been questioned (Campana and Nielson, 1985; Gunn *et al.*, 1992; Campana, 1999; Hedges *et al.*, 2004; Arslan, 2005; Miller *et al.*, 2006). Indeed, the assumption that the composition of the otolith cannot change significantly when it is *in situ* in the fish just prior to removal or after extraction when it is subject to analysis may be incorrect (Proctor and Thresher, 1998). The possibility of micro-chemical alterations such as leaching or contamination of otoliths during the extraction process has been highlighted as a point of concern (*e.g.* Proctor and Thresher, 1998; Hedges *et al.*, 2004).

The possible effects of trace elements leaching from these calcium carbonate aragonite structures prior to extraction and the possible labile nature of some Group II alkaline-earth metals (*e.g.* Mg) combined with the effects of *post mortem* handling have been little studied. The effect on otolith microchemistry of how the fish are captured, the problems associated between the time of capture and when they are dispatched (as reported from a study on deep sea fish species; Longmore *et al.*, 2011) and the time which they are stored frozen (*e.g.* days – months – years) prior to otolith extraction have also been little studied. Some authors indicate the importance of otolith removal directly over storage of the whole fish carcasses (prior to otolith removal) or the storage of the otoliths themselves (see Milton and Chenery, 1998; Rooker *et al.*, 2001; Brophy *et al.*, 2003; Swan *et al.*, 2006) due to the possible chemical composition being compromised after the capture of the fish (Proctor and Thresher, 1998; Hedges *et al.*, 2004).

1.7. Otolith microchemistry: Temporal stability

One of the main applications of otolith microchemistry has been to distinguish between water masses (both between and within freshwater, estuarine and marine water masses) that differ in their chemistry over spatial scales (Gillanders and Kingsford, 1996; Thorrold *et al.*, 1998*b*; Gillanders and Kingsford, 2000). These studies have focused on the movement patterns of fish between estuarine and marine waters (Gillanders, 2005) and in observing anadromy movements of fish from freshwater environments to marine (Kalish, 1990). However, interest in the movement of fish in freshwater and their migration to estuarine / marine environments is growing (*e.g.* Kennedy *et al.*, 1997; Walther and Thorrold, 2009; Ramsay *et al.*, 2011; Veinott *et al.*, 2012). Fish visiting or inhabiting water bodies that differ in their water chemistry will incorporate trace and ultra-trace elements from the surrounding water into their otoliths, picking up what can

be described as a unique chemical fingerprint for that moment in time. If we are to use spatial differences in water chemistry as a tool to look at origins or movement patterns of fishes then we need to have confidence that observed differences are maintained over time, *i.e.* exhibit temporal stability. However, water bodies are by their very nature highly dynamic environments with elemental concentrations differing considerably over small temporal scales *e.g.* as seen in estuarine environments (Elsdon and Gillanders (2006*b*). A number of studies have attempted to address the issue of temporal stability of otolith microchemistry but with limited success (see Elsdon *et al.*, 2008).

Most of these studies have tended to conduct their work over short term periods of $\leq$ 2 years (Edmonds *et al.*, 1992; Patterson *et al.*, 1999; Campana *et al.*, 2000). The exception is the study by Campana *et al.* (2000) whose research examined temporal stability over a longer time frame, 4 – 13 year intervals, but their time series analysis did not use a continuous data set with 4 locations resampled 5 times over a 13 year period. Milton *et al.*, 1997 found that temporal stability may have been a confounding issue when undertaking a study to identify the spawning estuaries of the tropical shad (*Tenualosa toli*) around the coastal seas off Sarawak and that further studies conducted over a greater time frame were required. Similarly, Walther and Thorrold (2008) using geochemical signatures contained within otoliths of an anadromous fish (American shad, *Alosa sapidissima*) found significant inter-annual variability between several rivers when looking at otolith $\delta^{18}O$ values. Few studies have attempted to look at the effects of inter-annual and intra-annual variability using biogeochemical analyses (Mercier *et al.*, 2011; Sturrock *et al.*, 2012). Clearly given the importance of understanding possible temporal effects and the stability of those chemical tags used in otolith microchemistry as a tool to understand movement patterns and origins of fish, more work is required to clarify and validate the use of this technique.

1.8. Otolith microchemistry: A tool to delineate between nurseries

For many marine fish species, the juvenile and adult phases of the life cycle can occupy very different environments that are geographically separate from each other (Brown, 2006*b*). Previous researchers have examined whether this has resulted in distinctive otolith elemental signatures for these different geographical areas that can be used to identify the origins of fish and thereby study their movement patterns and population

connectivity (Gillanders and Kingsford, 1996; Campana *et al.*, 1994; Gillanders and Kingsford, 2000; Geffen *et al.*, 2003; Gillanders, 2005; Brown, 2006*b*; Vasconcelos *et al.*, 2007; Elsdon *et al.*, 2008).

The changes in concentrations of various trace elements within the environment (*e.g.* marine / estuarine and freshwater or from food such as prey items) can influence the elemental concentration observed not only in the body tissues of the fish being studied, but also the concentrations that are incorporated into the calcium carbonate aragonite matrix of their otoliths (Campana, 1999; Geffen *et al.*, 2003; Gillanders and Kingsford, 2003). During the growth of the fish, elements contained within the water mass that surrounds the fish during its life are deposited within the structural layers of the otoliths. The uptake of the inorganic elements into the otolith stems from the surrounding medium (water), passes into the blood plasma via the gills and / or intestine and are then finally transported to the endolymph fluid which surrounds the crystallizing otolith (Campana, 1999; Wright *et al.*, 2002; Brown, 2006*b*). The incorporation of the various elements into the otoliths that occurs via the binding process of ions to proteins or assimilation between the spaces of the calcium carbonate and protein matrices (Gillanders and Kingsford, 2000) allows these structures to act as a long term data record of the environment(s) experienced by the fish. This has given rise to the use of microchemistry as a tool to try to identify the different geographical areas visited by the fish during its life, or to distinguish between fish reared on different nursery grounds and to identify the natal origin of fishes: all of these approaches having clear conservation and management implications (Geffen *et al.*, 2003). Furthermore, the use of otolith biogeochemistry has enabled scientists to differentiate between fish populations residing in adjacent freshwater catchments and fish which reside in different tributaries within a catchment during some part of their life history (*e.g.* Kennedy *et al.*, 1997; Walther and Thorrold, 2009; Ramsay *et al.*, 2011, 2012).

The use of otolith microchemistry has enabled some fish to be to be identified to their habitat of origin with a very high classification rate using statistics such as discriminant function analysis. For example, Vasconcelos *et al.* (2007) used the otolith chemical signatures to allocate five species of fish to their estuarine nurseries of origin along the Portuguese coastline with an accurate group classification rate of 77.1%, indicating the possible applications for this type on analysis in assigning adult fish to their estuarine nursery and in understanding connectivity between nursery grounds and coastal areas. A recent study by Fodrie and Herzka (2008) used otolith microchemistry to

identify movement patterns of juvenile flatfish and the contribution of nursery grounds within coastal embayments of southern California and Baja California, Mexico, with an accurate group classification rate between inner and outer zones of 61%, indicating the coastal embayments possessed distinct chemical fingerprints. Leakey *et al.* (2009) looked at the multi-elemental chemistry of juvenile sole (*Solea solea*), whiting (*Merlangius merlangus*) and sea bass in the Thames Estuary and identified differences in otolith chemistry between estuarine and coastal-captured fish. The differences between estuarine and coastally-caught fish were more evident in the less mobile sole compared to the more active whiting and bass. However, due to the physiological ability of the bass to migrate further up the estuarine environment, chemical signatures could be distinguished between the estuary-caught fish when looking at the salinity gradient change using Ba: Ca data.

Not only can the concentrations of naturally occurring elements within water masses (marine, estuarine and freshwater) be used to identify the origins of fishes, elements derived from anthropogenic activities can also be used to help distinguish between fish from different areas. For example, Geffen *et al.* (2003) studied the spatial differences observed in the otolith trace element concentrations of whiting (*Merlangius merlangus*) and plaice (*Pleuronectes platessa*) from five sites in the eastern Irish Sea (plaice) and from the eastern and western Irish Sea (whiting). Their results indicated that the observed spatial variation in otolith elemental concentrations were consistent with the distribution of contaminants observed around the Irish Sea by an earlier study by Leah *et al.* (1991, 1992*a*, *b*).

Further studies have shown that not only can the elemental composition of fish otoliths indicate regional signals but also the stable isotopic composition of the otoliths and scales can also indicate spatial variability and can be used to identify the origins of fishes. For example, Whitledge (2009) looked at the potential of using otolith chemistry to identify source environment for fishes in the upper Illinois River and Lake Michigan and to identify individuals that may have breached or circumnavigated electrical barriers in the Chicago Sanitary and the Ship Canal or had been transferred (via bait buckets) between those formally isolated drainages. Using both elemental and stable isotope otolith chemistry he found Lake Michigan fish were distinct from individuals sampled within the Illinois River and tributaries (using otolith $\delta^{13}C$) and fish collected from both the Fox and the Des Plaines Rivers could be distinguished not only from one another but from fish taken in the Illinois and Dupage Rivers (using Sr: Ca and Sr: Ba ratios). A more recent study has shown the use of $\delta^{13}C$ and $\delta^{15}N$ isotopes measured from scales of *S.*

trutta were marginally superior in their classification accuracy in identifying trout to their natal site on a small spatial scale compared to using multi-elemental signals obtained from scale hydroxyapatite and otolith aragonite within the River Dee catchment in North Wales (Ramsay *et al.*, 2012). The natural occurring variability observed in elemental concentrations recorded in otoliths combined with the use of microchemistry and isotopic analysis has shown increasing promise in helping researchers in identifying movement patterns over small spatial and larger geographical scales for fish within discreet water bodies. The continued use of these methods and the advances in technology can only strengthen our knowledge and understanding of fisheries movement and assist in future management of both stock and population structures.

1.9. Otolith microchemistry: Analytical techniques

The analysis of biogeochemical structures such as fish otoliths as tools to infer distinct connectivity patterns, stock structure and individual migrations for various fish species in both marine and freshwater environments has increased during the last decade (see Sturrock *et al.*, 2012). In order to quantify trace elemental concentrations a number of techniques have been used with some analytical methods being preferred more over others. Techniques have included electron microprobe energy-dispersive and wavelength-dispersive analysis, proton-induced X-ray emission, graphite furnace atomic absorption and micro-PIXE analysis (Sie and Thresher, 1992; Campana *et al.*, 1997; Proctor & Thresher, 1998; Campana 1999; Thresher 1999). However, the most commonly-used technique within this field of research is inductively-coupled plasma mass spectrometry or ICP-MS. This technique has grown in use partly due to its ability to detect elements at extremely low concentrations (parts per quadrillion – parts per trillion) allowing for a greater range and suite of elements to be precisely and accurately quantified (see Ludsin *et al.*, 2006 and references therein).

1.10. Otolith microchemistry: ICP-MS

The use of ICP-MS offers many benefits to researchers wishing to analyse trace and ultra-trace metals in either solutions (*i.e.* water) or solids (*e.g.* soils, rocks biological material), with most of the elements contained within the periodic table measurable (ICP-MS Technical Note, 2001). The use of ICP-MS achieves higher productivity and lower detection limits when compared to other systems and allows the multiple detection of numerous elements simultaneously, allowing rapid sample processing. To help understand the concept of ICP-MS and understanding its use in fisheries research a brief overview on how samples are introduced and how the ICP-MS works is presented.

Most samples which are analysed using ICP-MS are in solution form (*e.g.* digested material or liquids) which are then introduced into the plasma. However, solid samples can be analysed using ICP-MS by using heated cells or in most cases lasers which vaporize the sample material (similar to digesting a solid sample in solution-based analysis) which is then introduced into the plasma. Samples are first introduced to the plasma via a cross flow nebulizer (*i.e.* gas flow is at right angles to the flow of the sample) using a peristaltic pump system (see Figure 1.1). The nebulizer shears the liquid into vapor droplets forming an aerosol, with the now aerosolized sample droplets (at the correct size and velocity) pass through the spray chamber and on into the plasma.

The inductively-coupled plasma is generated by passing argon gas through a series of concentric quartz tubes and is energized by inductively heating the gas with an electrical radio frequency coil, making the gas electrically charged (containing both electrons and ion concentrations) at a temperature around 6000°C. The plasma gas is essentially neutral with positive ions almost all singly charged with equal amounts of both electrons and ions in the volume of the plasma. The liquid droplets containing the sample matrix and the elements we wish to assay are dried to form a solid then heated to form a gas.

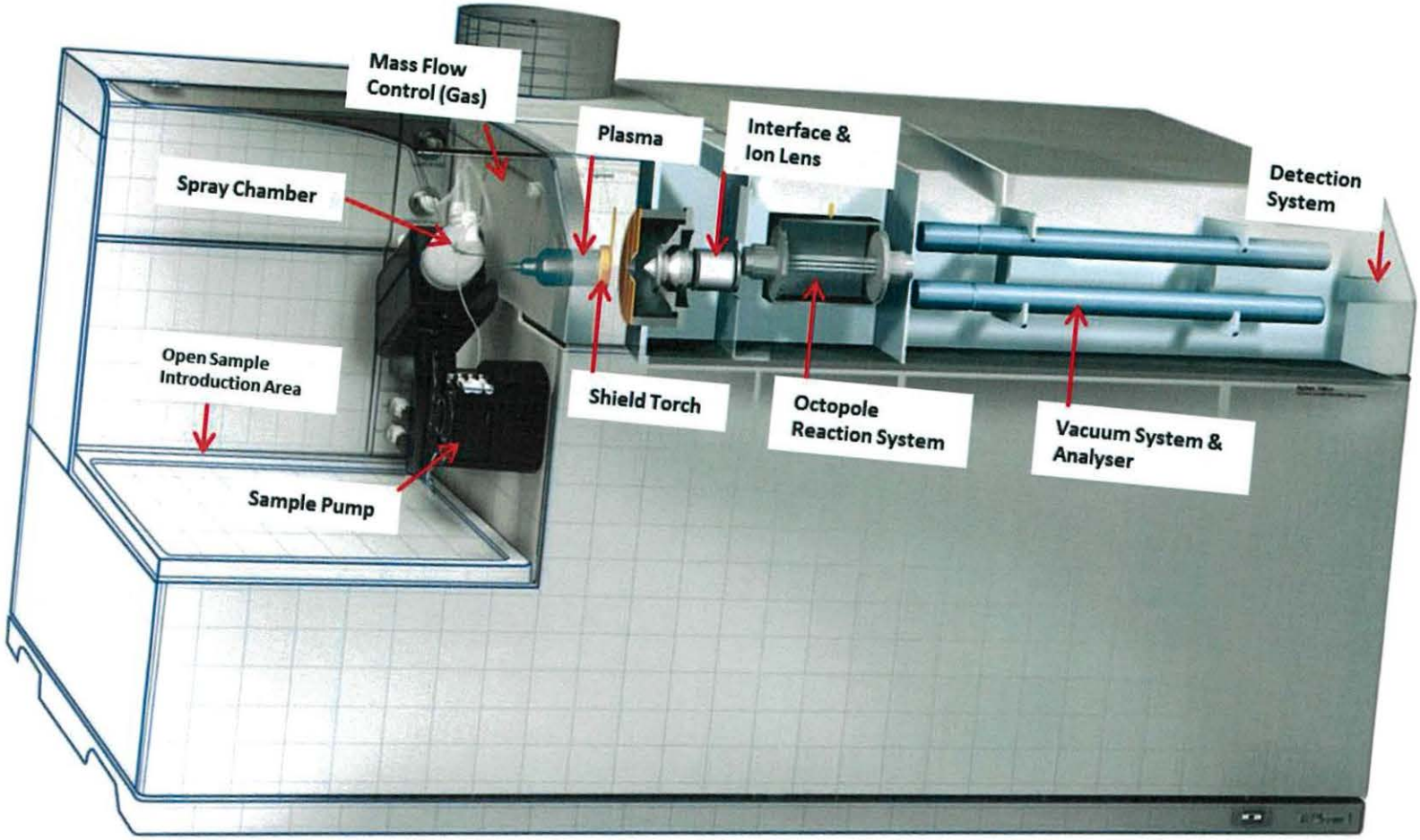


Figure 1.1. Layout of an inductively-coupled plasma mass spectrometer (ICP-MS), indicating sample introduction area and the identification of the components used in the analysis of both solution based and laser ablated material (Adapted from ICP-MS Technical Note, 2001).

The atoms travelling through the plasma absorb energy directly from the plasma and in doing so release an electron to become singly charged ions. At this point the singly charged ions enter the inter face region (Figure 1.1) where two cones (sampler and skimmer) each having an opening of 1 mm in diameter at their apex, sit a few millimetres apart and channel the sample towards the detector. To prevent any ions colliding with any gas molecules a vacuum is set up between the interface (cones) and the detector using a combination of turbomolecular pumps and roughing pumps creating a pressure or around 1×10^{-5} Torr.

Ion lenses positioned directly behind the interface focus the ions from the sample matrix towards the second generation octopole (Figure 1.1). Sample ions are nearly all positively charged and as such will repel other ions. A focusing lens created by passing the ions through a charged metal cylinder allows these positively charged ions to be focused into a focused ion beam which then passes into the octopole (the octopole consists of 4 rods approximately 20 cm in length and 1 cm in diameter). The ions are then separated in the quadrupole by their relative mass (*i.e.* mass filter) using the correct combination of radio frequencies and voltages between the four rods. Ions are then sorted out using their mass-to-charge ratio (m/z), with the selected m/z allowing only one mass measurement to pass through the detector (Figure 1.1) at any given moment. Ions exiting the octopole strike the surface of the detector (a series of dynodes) generating an electronic signal which is measured using the release of an electron each time an ion strikes, starting the amplification process and creating a cascade of electrons which in turn generates a measurable pulse. This measurable pulse allows the number of ions striking the first dynode to be counted and allows the identification of those elemental concentrations found within the sample to be identified. The ion counts measured by the detector are then transferred (as counts per second, cps) to the Agilent Proprietary ICPMS software (Agilent Technologies, U.S.A.), allowing the ICP-MS software to determine accurately how much of a specific element has been analysed in the material. This information is then extracted and subsequently transferred onto excel data spread sheets for the user to read and interpret the results.

1.11. Conclusion

The use of natural tags / markers (such as trace elements and stable isotopes) demonstrate great promise in determining movement patterns of fish populations and the possible identification of source environment for juveniles and adults that migrate or are transferred between areas. This has been facilitated by increased technological developments in stable isotopic research being more accessible (economically) to scientific researchers. The use of these chemical signatures may enable information to be gathered on the movement from specific habitats of juvenile fish and allow specific protocols for management and conservation efforts to focus on the protection of these habitats. Furthermore, studies must be focused on movement patterns to supplement data already gathered on density, growth and survival of juvenile fish in putative nursery areas enabling a greater rate of recruitment back to an adult population.

1.12. Aims of research for the thesis

The aims for the research of the current thesis are outlined as follows:

1. To determine whether pre-processing treatment of fish samples can influence the otolith microchemistry of fishes (Chapter 2). This aim will be achieved by examining how four common methods of dispatching fish (*i.e.* direct cull or using three types of anaesthetic: 2-phenoxyethanol, clove oil or MS-222), three of the most commonly used methods in transporting those fish samples (*i.e.* left fresh in air, on ice or in ice) and the period of storage duration (1, 5, 25 days and 6 and 12 months respectively) may affect the trace elemental concentrations of sea bass (*Dicentrarchus labrax*) otoliths.

2. To determine whether otolith elemental chemistry exhibits long-term temporal stability (Chapter 3). This aim will be achieved by examining intra-annual (*i.e.* within year) and inter-annual (*i.e.* between years) stability in the trace element chemistry in otoliths of juvenile plaice (*Pleuronectes platessa*) otoliths sampled from two recognised plaice nursery grounds along the North Wales Coast between 2004-2010.

3. Using brown trout (*Salmo trutta* L.) as a model species, to examine the efficacy of otolith microchemistry to identify natal river / geographical region of origin for juvenile brown trout (*Salmo trutta*) parr in the Irish Sea region and using this freshwater baseline to try to identify marine-caught adult sea trout back to geographical region of origin (Chapter 4).

Chapter 2

The effects of anaesthesia, transportation and storage on otolith microchemistry in juvenile European sea bass (*Dicentrarchus labrax* L.)

Abstract

It has been assumed that trace and minor-trace elements incorporated within the structures of fish otoliths are metabolically inert, with elemental compounds permanently accreted on the growing surface of these aragonite structures. However, methods used in the acquisition, transportation and retention of otoliths used in microchemistry analysis may introduce possible *post mortem* changes including loss or contamination and as such could bias the results obtained. To examine the possible effects of these protocols, sagittal otoliths of juvenile European sea bass (*Dicentrarchus labrax*) were subjected to the most commonly-used methods of dispatching fish (direct cull, 2-phenoxyethanol, clove oil, MS-222), transporting the samples (fresh, on ice, in ice) and finally storage of the whole fish frozen for 5 time periods (1, 5, 25 days and 6 and 12 months) prior to elemental determination using solution-based inductively coupled plasma mass spectrometry. Six elements - Na, Mg, K, Mn, Sr and Ba - were consistently found above detection limits and are focused on. No differences between the elemental chemistry of the left and right sagittal otoliths from the same fish were found for the six elements determined. Three elements - Na, Sr and Ba - indicated no significant changes in their concentrations in response to method of dispatch, transportation protocol or storage period. Significant differences in the elemental chemistry of Mg and K were observed for the storage period of 6 months compared to other time periods and for Mn between the storage periods of 1day and 12 months. These differences were attributable to a Type 1 error due to reduced variability in the Mg/K samples at 6 months and are not considered to be biologically meaningful. The results of this study indicate that the 4 killing methods used and the three methods of transportation do not significantly alter otolith chemistry for the 6 elements measured and that samples can be stored frozen *in situ* in fish for at least a year prior to measurement.

2.1. Introduction

The use of otolith microchemistry as a tool to discriminate between fish populations and determine stock structure (Thresher, 1999) for use in conservation ecology and fisheries management (Campana, 1999; DiMaria *et al.*, 2011; Ramsay *et al.*, 2011) has increased during the last decade (see reviews by Elsdon *et al.*, 2008; Sturrock *et al.*, 2012). Furthermore, its use in understanding the connectivity between local spawning grounds (Begg *et al.*, 1998; Edmonds *et al.*, 1999; Patterson *et al.*, 1999; Newman *et al.*, 2000) and nursery grounds (Thorrold *et al.*, 1998; Spencer *et al.*, 2000) has become a very valuable tool for fisheries scientists and managers alike. The relationship between the chemistry of the otolith and the water in which fish have resided has been used to understand the temperature and salinity histories of the oceans (Campana, 1999; Melancon *et al.*, 2009). Also, for many elements, their concentrations found in otoliths are thought to reflect the ambient water concentrations, albeit at differing degrees of mass discrimination (*e.g.* Ca, Sr and Ba; see review by Elsdon *et al.*, 2008 and references therein).

However, most studies using this type of chemical analysis to distinguish between fish from different populations or specific habitat types have utilized a variety of sample collection and storage methods, where either the whole fish or a sample, usually the removed paired sagittal otoliths, are stored for a period of time prior to analysis. It has often been assumed that the trace and minor-trace elements incorporated within the structures of fish otoliths are metabolically inert, with elemental compounds permanently accreted on the growing surface (see reviews by Campana, 1999; Elsdon *et al.*, 2008; Sturrock *et al.*, 2012). Furthermore, it is assumed that once accreted on the exterior surface elements are not reworked or re-absorbed (Campana and Neilson, 1985; Campana, 1999). However, some studies have indicated that many of the trace elements incorporated into the otolith matrix may not be bound in the calcium-rich inorganic matrix. For example, elements with a different valence or atomic mass to calcium may be attached to the various compounds within the organic (protein-rich) layers rather than bound within the calcium matrix itself (Dove *et al.*, 1996; Milton and Chenery, 1998; Proctor and Thresher, 1998). This could be problematic for those elements which are found to be more labile (see Hedges *et al.*, 2004) and not as tightly-bound to the calcium matrix as they may suffer differential diffusion out of the otolith into the surrounding medium (*e.g.* endolymph or preserving fluid such as ethanol) or contamination from the ethanol / endolymph (see

Gauldie *et al.*, 1998 for fluid migration in fish otoliths) may be absorbed back into the otolith (Milton and Chenery, 1998). If this is the case then detectable levels of those trace elements could change significantly in response to sampling and handling procedures, with these changes overemphasizing or indeed masking the actual influence the environment has on those elemental concentrations within the otolith (Milton and Chenery, 1998). Both *post mortem* handling and transportation combined with methods used to store samples for various periods of time could therefore influence data obtained when using inductively-coupled plasma mass spectrometry (ICP-MS) methods such as solution-based (sb) or probe-based methods *e.g.* laser-ablation (LA), micro-PIXE or electron microprobe analysis (see Table 2.1. and review by Elsdon and Gillanders, 2003). Moreover, some studies have observed differing results when comparing concentrations of various elements (*e.g.* Na, Mg, S, Cr, Zn and Ba) within the otoliths of fish species that have been stored frozen for differing periods of time (*e.g.* Rooker *et al.*, 2001; Brophy *et al.*, 2003; Swan *et al.*, 2006).

Table 2.1. Summary table of studies examining possible contamination effects observed in the elemental chemistry of fish otoliths using solution based (sb) or probe-based methods (*i.e.* laser ablation (LA), PIXE, micro-probe analysis) ICP-MS.

ICP-MS Method	Effects	Species	Elements Analysed	Method Effect	Significant Differences	Source
***Solution based* (sb)**						
	Handling & Storage Frozen (10 m)	*Coryphaenoides rupestris* *Helicolenus dactylopterus* *Merluccius merluccius*	Li, Mg, Ca, Mn, Cu, Rb, Sr, Ba	Metal & Plastic forceps Storage in paper envelopes Freezer Storage	Li Li Cr, Ba	Swan *et al.*, 2006
	Storage (1-81 d)	*Stizostedion vitreum*	Sr, Ba	None	None	Hedges *et al.*, 2004
	Post Mortem Handling & Storage (3 & 7 d)	*Thunnus thynnus* *T. albacares* *T. atlanticus*	Na, Mg, K, Ca, Mn, Sr, Ba	*Post Mortem* storage	Na, Mg	Rooker *et al.*, 2001
	Handling & Preparation	*T. atlanticus*		Micro-milling Sectioning Embedding	Al, Cu, Zn, Pb Co, Cu Al, V, Mn, Co, Ni, Cu,Ga, Cd, Pb	Arslan & Secor, 2008
	Preparation	*Parma microlepsi* *Achoerodus viridis*	Mg, Ca, Ti, Mn, Fe, Co, Ni, Sr, Ba	None	None	Dove *et al.*, 1996
	Comparisons sb / LA	*Perca flavescens*	Mg, Zn, Sr, Ba	Handling	Mg, Zn	Ludsin *et al.*, 2006
***Laser Ablation* (LA)**						
	Post Mortem Handling & Storage (5 & 12 m)	*Clupea harengus*	Mg, Zn, Sr, Ba, Pb	*Post Mortem* contamination Freezer Storage	Mg, Zn, Ba, Pb Mg, Zn	Brophy *et al.*, 2003
	Storage (1 d)	*Temalosa toli*	Li, Na, Mg, Mn, Co, Sr, Ba	Post capture freezing	Na, Mg, Co, Ba	Milton & Chenery, 1998
	Comparisons sb / LA	*Perca flavescens*	Mg, Zn, Sr, Ba	Laser intensity*	Ba	Ludsin *et al.*, 2006
Other Analyses						
Electron-probe Microanalysis (ED & WD)#	*Post Mortem* Handling &Preparation	*Nemadactylus macropterus* *Hoplostethus atlanticus* *Rhombosolea tapirina*	Na, K, Ca, S, Cl, Sr	Ethanol Handling	Na, Cl, K Na, Cl, K, S	Proctor & Thresher, 1998
Micro-PIXE	Contamination		Zn	Adhering tissue	Zn	Sie & Thresher, 1992

*Not verified due to possible high Ba in the mounting glass. #ED: Energy-dispersive, WD: Wavelength-dispersive. Duration: d days, m months.

Another factor to be considered is the unknown (or non-standardized) methods which may be used by organizations such as government agencies to collect samples for third parties during their routine research / monitoring surveys. When conducted by the researcher concerned, sampling, transportation and storage of specimens are usually well organized. However, in some cases of "opportunistic sampling", the processes of acquiring fish specimens or otoliths are often not as regulated and there may be little control in how samples have been obtained (Milton and Chenery, 1998). For example, in fish microchemistry studies samples may have been obtained either fresh or frozen from commercial fisheries (Kalish *et al.*, 1996) or have been purchased from local markets (Milton and Chenery, 1997) with an unknown post-capture history. Various methods of preservation and storage may also be implemented for the samples prior to analysis. For example, samples may be stored either by freezing the whole fish (Geffen *et al.*, 2003; Cook, 2011; Zeigler and Whitledge, 2010), or if freezer storage space is limited only the heads may be retained (Swan *et al.*, 2006). Solutions such as ethanol may also be used as a storage / preservation medium for either the whole or part of the fish (Milton and Chenery, 1998; Swearer *et al.*, 2003: Hedges *et al.*, 2004). One of the most commonly used methods for storage of fish otoliths is the use of dry paper envelopes (Kalish *et al.*, 1996; Swan *et al.*, 2006), usually prior to ageing. This method allows long-term and efficient storage (with regards to space requirements) of otoliths and reduces the requirement for whole fish samples to be retained either fresh or frozen due to the removed otoliths being stored completely dry. A similar method commonly used by researchers conducting microchemistry is to store extracted otoliths within pre-acid washed plastic vials or centrifuge tubes (Wright *et al.*, 2010; Cook, 2011).

A factor to be considered when analyzing the elemental composition of otoliths using solution-based (sb) or laser-ablation (LA) ICP-MS are the methods / techniques of extraction used by research scientists. These can vary from one scientific laboratory to another and problems can be compounded by access to the correct equipment necessary when extracting those otoliths for use in microchemistry. Most procedures used in the removal of otoliths for elemental analysis using ICP-MS involve using non-metallic (Thorrold *et al.*, 1997; Zeigler and Whitledge, 2010) or plastic forceps / equipment (Kalish, 1989; Brophy *et al.*, 2004; Miller *et al.*, 2005), or in some cases glass probes (see Gillanders and Kingsford, 1996; Hedges *et al.*, 2004). The use of these non-metallic instruments removes the possibility of significant surface contamination (through transference), resulting in possible increased trace and ultra-trace elemental concentrations

above their natural concentrations. In addition, the use of these types of apparatus / equipment (plastic or glass) also allows the user to pre-clean the equipment in various solutions of acid at differing concentrations (for examples of differing cleaning protocols see Milton and Chenery, 1998; Rooker *et al.*, 2001; Brophy *et al.* 2003; Vasconcelos *et al.*, 2007; Patterson *et al.*, 2009) prior to otolith extraction to avoid any potential metal contamination. Other methods employed in the extraction of otoliths have been the use of scalpels to expose the otoliths inside the cranial cavity of the fish and then their subsequent removal using ceramic forceps (see Fodrie and Herzka, 2008), enamel-tipped forceps (Wright *et al.*, 2010) or fine-tipped tungsten probes (Cook, 2011).

Thus, the techniques / methods of obtaining and storing whole fish or part samples (*e.g.* head) and methods of otolith extraction are potentially beyond the control of the researcher if samples are supplied from other sources and these factors may ultimately influence the chemical composition of the otoliths when measured (see Proctor and Thresher, 1998). Research scientists who may also have limited funds or who may be "working in remote areas" (Milton and Chenery, 1998) have probably the least flexibility in where their samples are obtained from and how they are stored. Indeed, it is not always feasible to remove directly the fish's otoliths after capture or in reality freeze the whole sample rapidly if the research is being conducted in more remote regions such as research conducted in tropical areas (with no / limited access to equipment to freeze or to store the samples; Hedges *et al.*, 2004). The issue of sample collection can be further confounded by the unknown amount of time the fish (whole or just the head) or indeed the otolith has remained in storage since its capture by collaborating third party research partners (Milton and Chenery, 1998).

The impacts of sample history prior to analysis on the accuracy of the measurement may be dependent on the element under consideration. The understanding by some authors is that the Group II alkaline-earth element/s (*e.g.* Sr, Ba) are more resilient to the effects of handling, storage and cleaning protocols because the elements are assumed to directly substitute for calcium within the crystal lattice structure of the otoliths (Milton and Chenery, 1998; Proctor and Thresher, 1998; Hedges *et al.*, 2004). In doing so, the element/s in question are therefore assumed to be tightly-bound within the crystal lattice structure and would therefore not be differentially leached out with respect to calcium. However, Campana (1999) and Rooker *et al.* (2001) have both indicated the possible effects of analytical handling procedures and their subsequent effect on the measurements of a range of elements in fish otoliths. Clearly if we are to have confidence in the results

obtained using ICP-MS involving trace and ultra-trace elements and to then utilize these results in stock discrimination, we must have confidence in the values obtained from the analysis of those samples and also to be able to state with some confidence they are not being confounded by issues that may have arisen from handling, *post mortem* changes and possible storage / extraction methods (Milton and Chenery, 1998; Swan *et al.*, 2006).

2.1.1. Aims

The aims of this study are outlined as follows:

1. To determine whether the methods used in dispatching fish (direct cull or using three types of anaesthetic: 2-phenoxyethanol, clove oil or MS-222) would affect the trace elemental concentrations in fish otoliths.

2. To determine whether the methods by which fish samples collected in the field are transported to the laboratory (*i.e.* either left fresh, on ice or in ice) would influence the trace elemental concentrations of the extracted otoliths from the fish.

3. To determine whether storage duration, chosen to represent short and long-term freezer storage of the whole samples (1, 5, 25 days and 6 and 12 months respectively), would affect the concentrations of trace elements in fish otoliths. Juvenile sea bass *Dicentrarchus labrax* was used as a model for this study.

2.2. Methodology

2.2.1. Hatchery specimens

A total of three hundred and ten 1 year old European sea bass (*Dicentrarchus labrax*) were obtained from the Bluewater fish farm at Penmon, Anglesey (BWFF, Selonda UK) on 24th March 2010. All fish were collected from the same enclosure tank, were subjected to the same constant environmental conditions (water temperature 25°C, salinity 36 ‰) and were fed the same diet 4-5 times a day.

2.2.2. Equipment preparation

All instruments used in the removal and storage of the otoliths (*e.g.* plastic-tipped forceps, glass beakers, petri dishes, 1.5 ml polypropylene micro-centrifuge tubes) were acid-washed in 10% nitric acid (see equipment preparation methods Appendix A).

To prevent the risk of zinc contamination due to aerosolized zinc particles derived from the glove powder (see Batley, 1989, Friel *et al.*, 1996; Dugan *et al.*, 2008) powder-free nitrile gloves (Fisherbrand) were used during all handling, extraction and storage procedures of all samples.

Prior to the initial digestion of the right sagittal otolith, equipment used *e.g.* ICP-MS tubes and pipette tips were acid washed using acid-washing protocols implemented at the British Geological Survey (B.G.S) (for details see Appendix A).

2.2.3. Method of despatch

The juvenile sea bass were euthanized on site according to Home Office Schedule 1 method using either i) a sharp blow to the head, followed by destruction of the brain and transection of the spinal cord or ii) overdose in a strong solution of one of the three most commonly anaesthetics in fish biology studies (tricaine methanesulfonate (MS-222), clove oil, 2-phenoxyethanol) followed by destruction of the brain and transection of the spinal cord. The volume and solution concentrations are described in more detail in Appendix B.

2.2.4. Direct dissection, cleaning procedures and sample retention

As a control, 10 bass were killed immediately on site at BWFF and the left and right sagittal otoliths were extracted (direct removal group) from the cranial cavity using acid-washed plastic forceps (see Appendix A for acid washing protocols). Both the left and the right sagittal otoliths were placed into a petri dish containing Millipore™ ultra-pure Milli Q water (hereafter referred to as Milli Q) and cleaned of any adhering vestigial tissue using an acid-washed fine-bristled nylon brush and then triple-rinsed in Milli Q. The left and right otoliths from 5 fish were stored in dry envelopes and the left and right otoliths of the remaining 5 control fish were stored separately in labelled acid-washed 1.5 ml polypropylene micro-centrifuge tubes, sealed and transported to a laminar positive flow cabinet where they were dried for a period of 24 hours prior to storage.

2.2.5. Transportation and storage of otoliths prior to ICP-MS

The juvenile sea bass were stored for transportation to the School of Ocean Sciences using three portable storage containers and three different cooling methods (ambient temperature, on ice, in ice). These were chosen to represent the methods most likely used by fisheries research scientists in the field to preserve their samples for transportation to their permanent storage in the laboratory. To enable the temperature to be monitored throughout the transportation of the sea bass samples to the laboratory, three portable electronic thermometers were attached to the outside of the three transportation containers with the thermal sensor then deployed inside each of the containers. The temperatures were recorded before the commencement of the journey from the fish farm to the School of Ocean Sciences and once again before the fish were placed into the freezers for the storage experiment. The thermal sensors were then used to record (daily) the temperature of the storage freezer for the duration of the experiment. The sampled fish were transported in bags either (1) fresh (no ice), (2) left on ice or (3) packed in ice (ice place beneath, around and over the fish samples). Storage method one required the samples (n =100) to be placed in labeled polythene bags and left in a polypropylene storage container for the duration of the sample collection at ambient temperature recorded as 20.3°C (±0.2 °C). For the second storage method, fish samples (n =100) were placed in the labeled polythene bags and stored directly on ice at a temperature recorded at 11.6°C (±0.1°C) whilst transported to the fisheries laboratory. For the final storage method fish samples (n

=100) were placed in labeled polythene bags and stored in ice (contained within a polystyrene box) at a temperature recorded at -0.1°C (±0.1 °C). To test the hypothesis that the storage period may influence the chemical composition of juvenile sea bass otoliths, a storage protocol was set up whereby fish obtained from BWFF were stored frozen for varying lengths of time prior to analysis. The time periods chosen to store the sea bass were seen as being representative of fish samples stored for short-term research work (*i.e.* undergraduate and postgraduate project work); these time periods were 24 hours, 5 days, 25 days, 6 months and 12 months respectively.

2.2.6. Extraction of otoliths after storage period

After the required period of storage duration had elapsed, bass samples were removed from the freezer (in batches of 25) and were allowed to thaw for approximately 20 minutes to allow the brain case to defrost prior to their otoliths being removed. Length (to the nearest 0.1cm) and weight (to the nearest 0.1g) of each fish were taken just prior to otolith extraction. Both left and right sagittal otoliths were extracted from the cranial cavity of the sea bass using acid-washed fine-tipped plastic forceps placed into labelled left and right acid-washed petri dishes containing Milli Q and cleansed of any adhering vestigial tissue using the methods previously described. The left and right otoliths were then stored in separately-labelled acid-washed 1.5 ml polypropylene micro-centrifuge tubes, sealed and transported to a laminar positive flow cabinet where they were dried for a period of 24 hours. To ensure the removal of any possible remaining vestigial tissue or detritus trapped within the interstitial lamellar spaces (see Brophy *et al.*, 2003 on contamination residue within microscopic calcified structures) both otoliths were subjected to 5 minutes sonication in 3% H_2O_2 (see Appendix A for detailed cleaning methods), triple-rinsed in Milli Q and dried for a further 24 hours in the laminar positive flow cabinet.

2.2.7. Otolith dilution (Part I)

Right sagittal otoliths were used in all the analyses using sb-ICP-MS, unless for reasons otherwise stated (*e.g.* the right otolith being crystalline or lost during sample extraction / preparation or to compare right with left). The methods used to dissolve the right sagittal otoliths in preparation for ICP-MS is a two stage process, with the acid (HNO_3 / HCl) concentrations required to dissolve the whole otoliths calculated from the concentration of

Ca determined from the stoichiometry of aragonite *i.e.* 400,000 μg g^{-1} (see Dove *et al.*, 1996; Milton and Chenery, 1998; Zdanowicz, 2001; Ludsin *et al.*, 2006; Swan *et al.*, 2006; Lowe *et al.*, 2011). The first stage involves the initial pipetting of a 2 ml solution of 16.7% HNO_3, and 6.4% HCl allowing time for the otolith to effervesce (a reaction of the calcium carbonate and the acid). Once this initial effervescing has subsided, a second 2 ml of 16.7% HNO_3, and 6.4% HCl solution is then pipetted into the partly dissolved otolith sample ensuring that any residue that may have effervesced up around the internal part of the ICP-MS tube is rinsed back into the bottom of the tube. A further 20 minutes is allowed for the digestion of the otolith and to allow the effervescing to subside, before the otolith-acid solution was brought to a volume of 10 ml by adding 6 ml of Milli Q (taking care to rinse around the internal part of the ICP-MS tube).

2.2.7.1. Otolith dilution (Part II)

Following the initial dilution of the otolith samples, the calcium carbonate ($CaCO_3$ as aragonite) within the digestion otolith-acid solution is still at a concentration too strong to assay on the ICP-MS and so requires further dilution. If the otolith solution were to be introduced into the ICP-MS and run after the first dilution stage possible interferences due to instrument drift or distortions to elemental measurements caused by high concentrations of elements (*e.g.* Na in sea water or Ca in otoliths) within the matrix could occur. This would result in the formation of deposits on instrument components (*e.g.* the skimmer and both sampler interface cones) causing potential changes to the profile of the sample cone orifice and resulting in possible signal suppression or enhancement of elements at lower concentrations (Beauchemin *et al.*, 1988; Jarvis *et al.*, 1992; Dove *et al.*, 1996; Dove and Kingsford, 1998; Swan *et al.*, 2006). Therefore, the sample was diluted further by pipetting 0.25 ml into an ICP-MS tube and adding 4.75ml of 1 % HNO_3 / 0.5% HCl to bring the combined otolith-acid solution to a total volume of 5 ml and this dilution was run through the ICP-MS. For the typical otolith weights in this study, this would give a Ca concentration between 100-200 mg/L the optimal balance for the B.G.S ICP-MS between cone clogging, signal suppression enhancement and detection limits (S. Chenery *pers. comm.* 2013).

2.3. sb-ICP-MS data processing

2.3.1. Data screening

The assessment of which elements to measure in the juvenile bass otolith were decided in discussions with Dr. S. Chenery (Co-supervisor, B.G.S) after reviewing published data on microchemistry of fish otoliths.

In total 29 isotopes were targeted for measurement using sb-ICP-MS: Li, Na, Mg, Al, K, Ca [42, 44], Sc, Mn, Fe, Cu, Zn, Ge, As, Rb, Sr, Rh, Cd [111,114], In, Sn, Te, Ba, La, Ir, Pb [206, 207, 208], U. These elements were chosen to run in the ICP-MS because of (1) the ease with which those elements could be assayed and (2) the sensitivity (with regards to limits of detectability) of the mass spectrometer to determine their strength (concentration) and quality (limits of detection, LOD). Scandium, Ge, Rh, In, Te, and Ir were all added as internal standard isotopes (see below). Multiple isotopes of lead were included as these may vary with natural abundance but may be corrected for by the instrument software. Microchemical analysis of all otolith samples (including internal standards, quality control standards and system blanks) were run on an Agilent series 7500 inductively-coupled plasma mass spectrometer equipped with an ASX 500 series auto sampler. Samples (including all standards) were injected and aerosolized in the ICP-MS using an argon gas plasma produced in a quartz torch at 5000° Kelvin, sample ions were then drawn from the plasma through sample and skimmer cones into a second generation Octopole Reaction System.

Elements were screened for the possible effects of poly-atomic ionization interference (see Gray, 1989; Evans and Ebdon, 1990) and the interferences caused by the use of hydrochloric acid [HCl] in otolith digestion and equipment preparation, were also considered. These possible interferences may come about through the use of hydrogen (H: 1.0 g/mol), oxygen (O: 15.9 g/mol), nitrogen (N: 14.0 g/mol) and argon (Ar: 39.9 g/mol) due to their high analyte matrix concentrations within the plasma while running the ICP-MS. Elements such as arsenic (As: 74.9 g/mol) and iron (Fe: 55.8 g/mol) have the potential to suffer from these possible interferences (when looking at their atomic weights) from ArCl (75.4 g/mol) or ArO (55.9 g/mol) respectively. Similar possible interferences can be observed when using hydrochloric acid in the cleaning processes and otolith digestion through the isotopic combinations in the form of ArCl and CaCl (75.5 g/mol).

Furthermore, some interference may come about when running the ICP-MS in the form of CaO (56.0 g/mol) when using a higher concentration rate of Ca in the standards (Dr. Chenery *Pers comm.*, 2009).

During analysis, raw data measurements from elements are corrected from instrumental sensitivity "drift" with time using the internal standards (ISTD) within the Agilent 7500 mass spectrometer integrated software. Normally the ISTD closest in mass to the isotopes of the elements of interest are used for correction as "drift" is typically mass dependent. The software also calculates the sensitivity of the instrument for the different isotopes of interest with the external concentration standards. These sensitivities are then applied to the sample measurements to derive elemental concentrations.

Two of the 23 elements analysed (Fe and As) were subsequently removed (see Table.2.2.) due to possible polyatomic ion interference (Dr. Chenery *Pers comm.*, 2010). In addition the Rare Earth element (REE) La was also omitted from the final data set due to its low concentration within the natural environment. (Dr. Chenery *Pers comm.*, 2010).

Table 2.2.The elements tested (n = 23) for in the initial raw data output for the sea bass otoliths in order of their relative atomic mass. Polyatomic and elements observed at their limits of detection (LOD) using a multi-element solution standard and internal standards of the ICP-MS during the sample analysis are indicated (*polyatomic elements, #elements exhibiting their LOD).

Elements				
$Li^{\#}$	K	$Cu^{\#}$	Sr	**La**
Na	$^{42,44}Ca$	$Zn^{\#}$	$^{111,114}Cd^{\#}$	$^{206,\ 207,\ 208}Pb^{\#}$
Mg	Mn	As^{*}	$Sn^{\#}$	$U^{\#}$
$Al^{\#}$	Fe^{*}	Rb	Ba	

Element in **bold** is a rare Earth element and would probably not be observed above the LOD within the samples (Dr. Chenery *Pers comm.*, 2010).

2.3.2. Analytical figures of merit including limits of detection

The limits of detection (LOD), alternatively known as detection limits (DL) are one of the measures of instrument performance and is the point where we can no longer statistically determine whether the concentration differs from zero. The use of the internal analytical run blanks enables their calculation. The DL values in the current study were calculated using the solution concentrations equivalent to three times the standard deviation of the blanks analysed. As the measured concentration of samples is reduced and approaches

zero, the relative standard deviation (RSD %) of repetitive analyses rapidly increases. An alternative definition of the DL is where the RSD % of repetitive analyses reaches 100%. Similarly we may define a quantification limit QL as the equivalent of ten times the standard deviation of the blanks response.

Subsequently, two elements subject to polyatomic interference (Fe and As) and those isotopes falling below the limits of detection (Li, Al, Cu, Zn, $Cd^{111, 114}$, Sn, $Pb^{206, 207, 208}$ and U) were omitted from any further analysis (Table 2.2.). In total eight elements were identified to be above the LOD: Na, Mg, K, Ca, Mn, Rb, Sr and Ba. However, one element (Rb) was observed to be below the LOD in the 184 day (6 months) samples; it should be noted that the detection limit is not a fixed value but will vary from analytical run to run as a result of either purity of digestion reagents, cleanliness of equipment or small difference in the way the ICP-MS is set-up. Accuracy and precision of analytical runs were calculated from the measurement of quality control solutions with elemental concentrations sufficiently away from the detection limit to avoid its influence.

2.3.3. Elemental outliers & corrections

The raw data output for each element was assessed for its mean, median, standard deviation and % relative standard deviation (%RSD). The %RSD is based on the replicate measurements of the calibration standards used in the analysis and reflects the level of precision which has been achieved for each of the elements assayed (Brophy *et al.*, 2003). %RSD values between 3-20% are considered optimal to include an element within the data analysis (Dr. Chenery *Pers comm.*, 2010). As noted above (2.3.2.) when measured concentrations begin to approach the detection limits, the %RSD will naturally increase, therefore the precision of elemental concentrations measured within samples (as observed from duplicate analyses) may be greater than that expected from the quality control solutions and exceed that desired for analysis (>20%). However, if the true variation in the otolith samples is greater than this analytical variation, useful data may still be obtained.

Furthermore, certain biological factors such as handling and *post mortem* effects (see Milton and Chenery, 1998; Swan *et al.*, 2006) could also influence the elemental concentrations within the bass otoliths and must also be taken into consideration. In each case, the erroneous data points within the sample must be (after careful consideration as to what their cause may likely to be; see Barnett and Lewis, 1994) either removed or assessed for their inclusion in the analysis as possible outliers identified using the method described

by Grubbs (1950, 1969) and adjusted by using a method known as Winsorisation (value set equal to the next lowest or highest value, see Hawkins, 1980; Sokol and Rohlf, 1995). After performing both the Grubbs and Winsorisation analyses on the eight elements (Na, Mg, K, Ca, Mn, Rb, Sr and Ba) to correct the already identified outliers observed in the original raw data set (see Appendix C), each of the seven elements were then normalised to calcium using element:Ca ratios (Campana, 1999; Thresher, 1999; Elsdon and Gillanders, 2004).

2.4. Statistical analyses

2.4.1. Data transformations

Element:Ca ratio data were subjected to both tests of normality and homogeneity of variance using Minitab v. 14. Due to the large numbers observed within the data set (n = 310) and the sensitivity of the Anderson-Darling test (AD) of normality where any slight deviation within a large data set may increase the chances of a rejection of a normal distribution (see McGuinness, 2002), the AD test was used to assess normality distributions visually (basic and graphical plots see D'Avignon and Rose, 2013; Gillanders and Kingsford, 2003). Tests of homoscedasticity were conducted using the Levene's test for equal variances (see Underwood, 1997; Gillanders and Kingsford, 2003; de Vries *et al.* 2005).

After Log_{10} transformation of the seven element:Ca ratios (Na, Mg, K, Mn, Rb, Sr and Ba), the assumptions of equal variance were met for five of the initial seven elements (Na, K, Mn, Sr and Ba) for the dispatch, transportation and storage methods. However, the element Mg indicated equal variance for the dispatch and transportation methods but failed the assumptions of equal variance when looking at the effects of storage duration. However, where data remained heterogeneous after transformation analyses (*e.g.* Mg and storage duration) analysis were still performed using a probability of $\alpha = 0.05$ (based on McGuinness, 2002) in which tests assessing homogeneity of variance were found to be more sensitive to violations of the ANOVA assumptions (*i.e.* homogeneity assumptions not being met before running ANOVA) than the actual ANOVA (see De Vries *et al.*, 2005).

Furthermore, ANOVA has been found to be robust to departures of normality and heterogeneity where data are balanced and sample numbers are shown to be relatively large (see Underwood, 1997; Gillanders and Kingsford, 2003). However, due to Rb failing to meet the LOD for the 6 month freezer storage period and being found to be biologically-mediated (found within animal tissue and in some occurrences mimicking potassium in its distribution and excretory patterns; Hays and Swenson, 1985; Soetan *et al.*, 2010), this element was removed from the statistical analysis. Furthermore, Rb has been shown to be present as single-valent inclusions in the otolith and thought (by some) in having limited biological relevance in microchemistry studies because of its extremely labile nature (Rooker *et al.*, 2001).

2.4.2. Statistical analysis

Both univariate and multivariate parametric and non-parametric analyses were used to assess differences in trace and multi-elemental elemental concentrations. Multivariate analysis of variance (MANOVA) was performed on the element:Ca ratio data for each of the four euthanasia, three transportation and five freezer storage methods to evaluate any changes in the elemental concentrations of the otoliths. Individual ANOVAs were run in conjunction with the MANOVA to identify any possible changes in elemental concentrations between methods using a Bonferroni *post-hoc* analysis. Finally a 3-Way ANOVA was run to assess each of the methods and the interactions between methods for possible changes in elemental concentrations of the otoliths.

2.4.3. Assessment of right and left otoliths

To determine whether right or left sagittal otoliths differ in their elemental concentrations (Campana *et al.*, 2000; Secor *et al.*, 2001) and to identify whether either the right or left sagittal otolith could have been used for the experiment, 45 randomly chosen left otoliths from the remaining samples were run on the ICP-MS. The six elements meeting the criteria for their LOD and %RSD (Na, Mg, K, Mn, Sr and Ba) were then statistically tested using a paired *t*-test against their opposite right otolith.

2.5. Results

2.5.1. Assessment of right and left otoliths

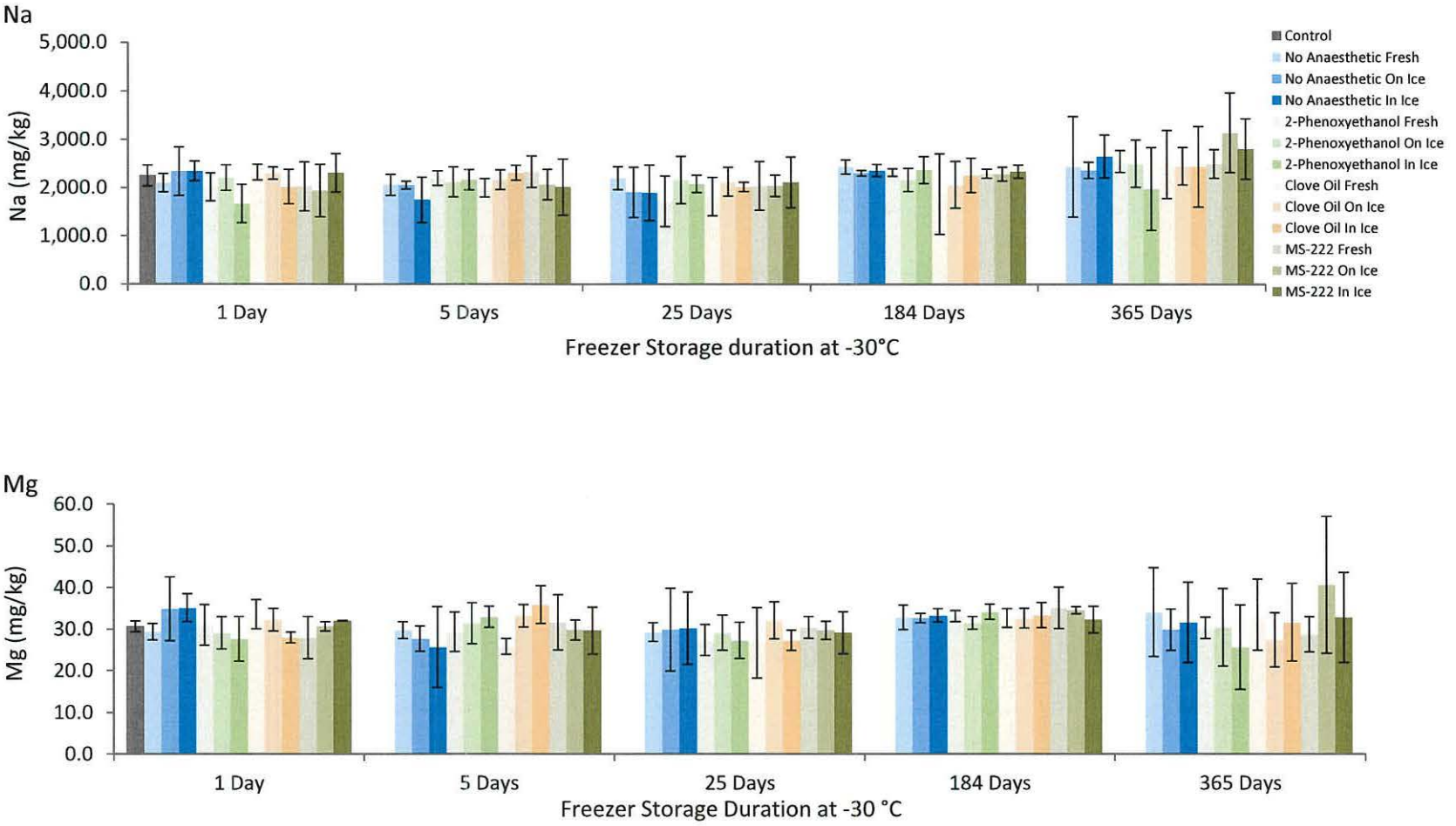
Results of the paired *t*-test analyses showed no significant differences (all $P > 0.05$) in elemental concentrations between either the left or right otoliths for the six elements examined (Table 2.3.).

Table 2.3. Comparisons between the right and left sagittal otoliths of juvenile sea bass, measuring their elemental concentrations as element: Ca ratios ($x10^{-3}$) using a paired *t*- test. Left and right otoliths are shown as mean ($\bar{x}$) ± 1 standard deviation (sd).

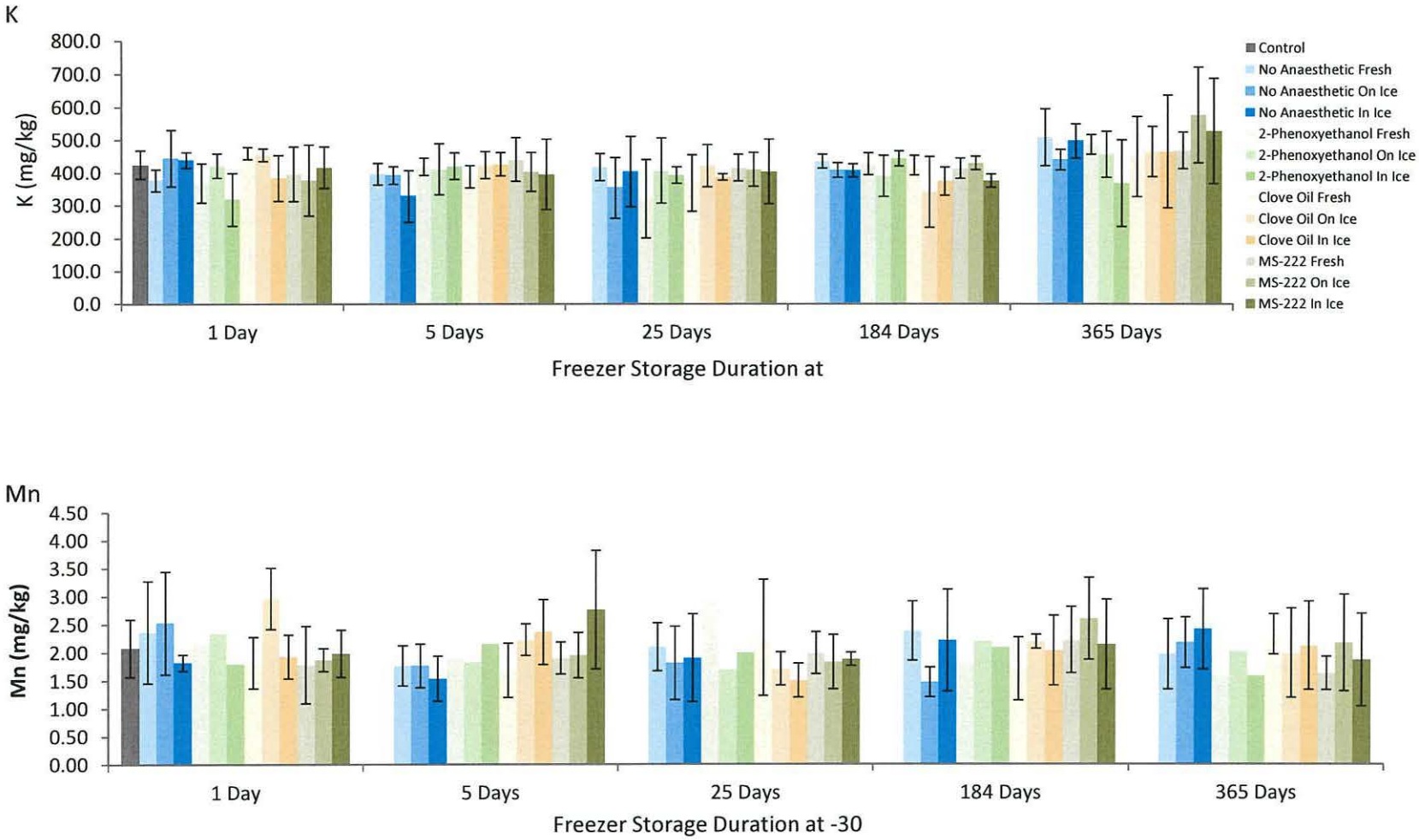
		Left Otolith	Right Otolith		
Element	**n**	$\bar{x} \pm$ sd	$\bar{x} \pm$ sd	***t***	***P***
Na	45	7.139 ±0.458	7.219 ±0.781	0.59	0.555
Mg	45	0.111 ±0.019	0.118 ±0.023	1.69	0.098
K	45	1.285 ±0.071	1.339 ±0.179	2.05	0.050
Mn	45	0.006 ±0.001	0.007 ±0.003	1.39	0.171
Sr	45	3.990 ±0.217	4.156 ±0.599	1.84	0.072
Ba	45	0.005 ±0.001	0.005 ±0.001	1.48	0.147

2.5.2. Analysis of 1, 5, 25 day, 6 and 12 month freezer storage samples

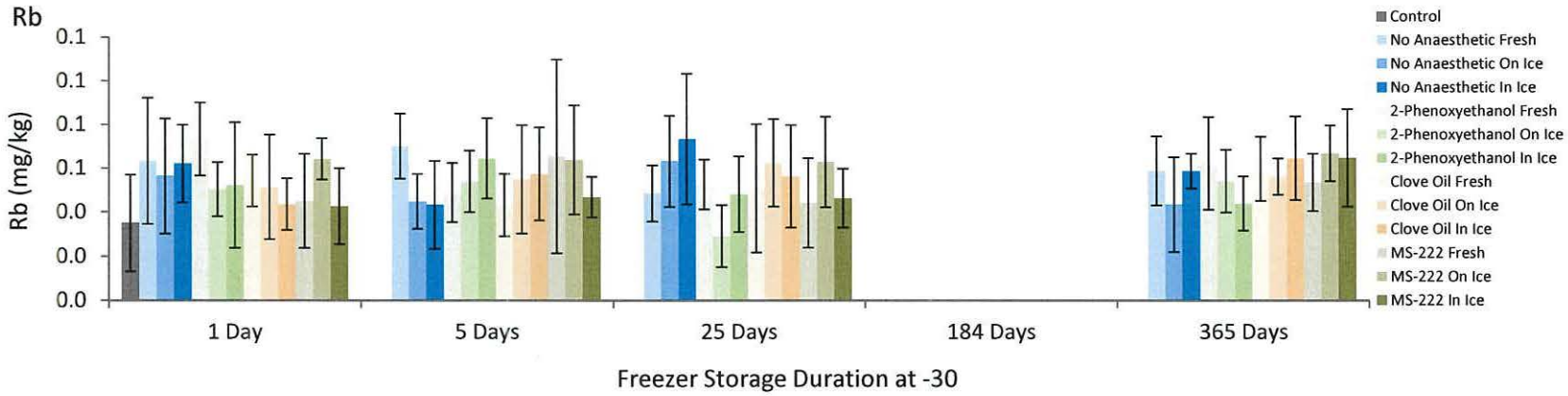
The mean (±sd) otolith elemental concentrations (expressed as absolute concentrations mg/kg) for each of the seven elements are presented in Figure 2.1. to provide a visual assessment of the effect of methods of dispatch and transportation and storage duration on otolith microchemistry. To distinguish further the possible variations observed in Figure 2.1., the mean ± standard deviation values are tabulated in Table 2.4.



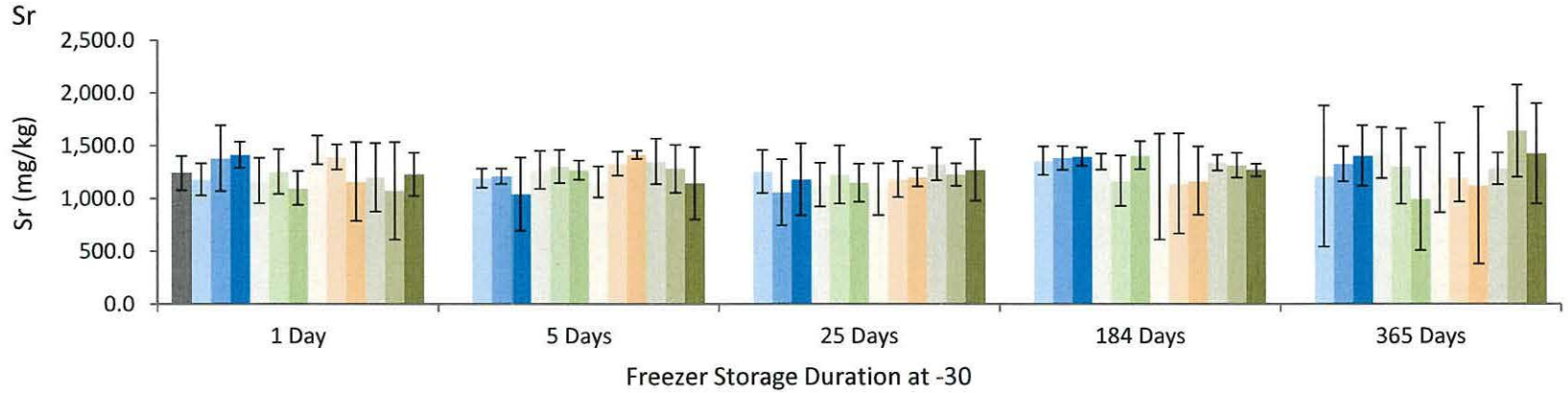
(Figure 2.1. and caption are continued overleaf)



(Figure 2.1. and caption are continued overleaf)



Note: When looking at the bar chart for Rb there is no 6 month (184 days) data presented as these samples were found to be below the LOD.



(Figure 2.1. and caption are continued overleaf)

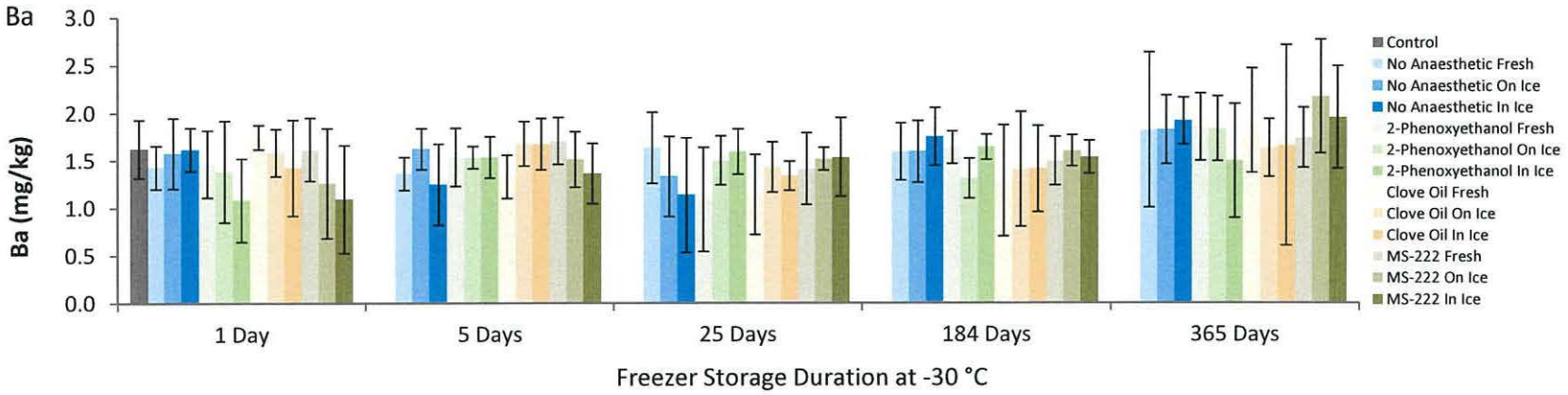


Figure 2.1. The effect of method of anaesthesia, transportation method and storage duration on elemental concentrations of Na, Mg, K, Mn, Rb, Sr and Ba (mg/kg) in juvenile sea bass (*D. labrax*) otoliths. Data are presented as mean values ±1 sd, Each anaesthetic method totalled 5 fish (60 fish / storage duration) with the exception of the control (n = 10).

Table 2.4. The effects of method of anaesthesia, transportation method and storage duration on element concentrations of Na, Mg, K, Mn, Rb, Sr and Ba (mg/kg) in juvenile sea bass otoliths. Each anaesthetic method totalled 75 fish (25 fish / transport method) with the exception of the control (n = 10).

Na (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184	365
Direct Cull	Fresh	2102.3 ±192.4	2064.4 ±219.2	2203.3 ±239.3	2442.2 ±147.5	2446.9 ±1042.7
	On Ice	2340.9 ±501.3	2056.9 ±79.7	1910.2 ±522.3	2312.9 ±58.3	2374.2 ±169.8
	In Ice	2350.1 ±205.6	1752.8 ±470.2	1900.7 ±579.4	2367.9 ±124.6	2662.3 ±446.0
2-phenoxyethanol	Fresh	2015.7 ±290.8	2202.6 ±151.4	1724.4 ±527.7	2328.0 ±79.6	2562.3 ±227.6
	On Ice	2211.0 ±265.3	2128.2 ±311.9	2169.1 ±490.2	2175.3 ±242.1	2511.2 ±489.1
	In Ice	1672.1 ±398.1	2172.9 ±211.9	2086.1 ±182.3	2380.8 ±282.1	1985.2 ±861.3
Clove Oil	Fresh	2325.3 ±162.5	1999.7 ±191.8	1823.9 ±396.7	1879.6 ±839.1	2499.3 ±709.2
	On Ice	2305.9 ±127.2	2171.1 ±207.2	2133.0 ±301.0	2074.7 ±487.7	2462.6 ±388.7
	In Ice	2023.9 ±360.6	2315.4 ±156.5	2020.6 ±583.5	2269.1 ±361.4	2448.1 ±838.5
MS-222	Fresh	2029.9 ±509.0	1943.2 ±546.4	2309.2 ±399.4	2305.7 ±94.0	2508.2 ±295.6
	On Ice	2337.9 ±326.0	2072.6 ±318.4	2020.6 ±583.5	2293.5 ±146.3	3149.8 ±823.3
	In Ice	2051.5 ±506.4	2048.4 ±220.6	2122.8 ±527.5	2346.6 ±136.6	2816.6 ±631.1
Control	None	2253.8 ±219.6	-	-	-	-

Mg (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184	365
Direct Cull	Fresh	29.4 ±2.0	29.7 ±2.0	29.3 ±2.2	32.8 ±3.0	34.1 ±10.7
	On Ice	34.9 ±7.7	27.7 ±3.1	29.9 ±10.0	32.7 ±1.2	29.9 ±5.0
	In Ice	35.2 ±3.4	25.7 ±9.7	30.2 ±8.7	33.3 ±1.6	31.6 ±9.7
2-phenoxyethanol	Fresh	31.0 ±4.9	29.3 ±4.8	27.4 ±3.7	33.2 ±1.3	30.3 ±2.6
	On Ice	29.1 ±3.9	31.4 ±5.0	29.1 ±4.3	31.5 ±1.5	30.5 ±9.3
	In Ice	27.7 ±5.4	33.0 ±2.5	27.3 ±4.4	34.2 ±1.8	25.7 ±10.2
Clove Oil	Fresh	33.6 ±3.6	25.9 ±1.9	26.7 ±8.5	32.7 ±2.2	33.5 ±8.6
	On Ice	32.3 ±2.7	33.2 ±2.7	32.1 ±4.5	32.6 ±2.4	27.5 ±6.5
	In Ice	28.0 ±1.3	35.9 ±4.6	27.3 ±2.4	33.4 ±3.0	31.7 ±9.4
MS-222	Fresh	27.9 ±5.1	31.6 ±6.7	30.4 ±2.6	35.1 ±5.0	28.8 ±4.3
	On Ice	30.7 ±1.1	29.8 ±2.4	29.7 ±2.2	34.6 ±0.9	40.6 ±16.5
	In Ice	32.0 ±0.0	29.7 ±5.7	29.1 ±5.1	32.3 ±3.2	32.8 ±10.9
Control	None	30.6 ±1.3	-	-	-	-

K (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184	365
Direct Cull	Fresh	377.4 ±34.3	396.9 ±34.1	417.5 ±41.4	436.5 ±20.8	509.4 ±86.4
	On Ice	444.8 ±86.9	392.9 ±27.9	356.6 ±93.4	409.3 ±23.1	441.0 ±31.0
	In Ice	439.5 ±23.3	329.6 ±78.5	403.9 ±107.3	408.6 ±20.5	498.4 ±52.2
2-phenoxyethanol	Fresh	369.7 ±60.4	419.4 ±26.7	321.3 ±120.0	428.0 ±33.7	487.4 ±29.8
	On Ice	422.2 ±37.2	411.4 ±77.9	406.9 ±99.5	390.9 ±63.4	457.1 ±70.5
	In Ice	319.3 ±80.4	420.7 ±40.4	393.5 ±25.6	444.8 ±23.2	369.0 ±131.7
Clove Oil	Fresh	460.3 ±18.2	388.7 ±34.8	369.2 ±86.6	423.8 ±30.1	450.1 ±122.8
	On Ice	455.1 ±18.8	423.7 ±41.5	387.4 ±64.8	342.5 ±108.1	465.4 ±76.6
	In Ice	383.6 ±70.2	426.7 ±35.8	387.4 ±10.0	375.4 ±43.7	465.6 ±172.2
MS-222	Fresh	396.8 ±83.6	441.5 ±66.9	416.0 ±41.2	414.5 ±31.3	469.6 ±55.4
	On Ice	377.6 ±108.0	403.0 ±59.9	411.1 ±52.3	431.4 ±20.6	577.9 ±145.1
	In Ice	416.7 ±63.6	395.4 ±107.8	404.5 ±99.0	376.2 ±20.7	528.1 ±160.8
Control	None	425.3 ±43.6	-	-	-	-

Table 2.4. Continued.

Mn (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184	365
Direct Cull	Fresh	2.4 ±0.9	1.8 ±0.4	2.1 ±0.4	2.0 ±0.5	2.4 ±0.6
	On Ice	2.5 ±0.9	1.8 ±0.4	1.8 ±0.7	1.5 ±0.3	2.2 ±0.5
	In Ice	1.8 ±0.1	1.5 ±0.4	1.9 ±0.8	2.2 ±0.9	2.4 ±0.7
2-phenoxyethanol	Fresh	2.1 ±0.2	1.9 ±0.4	2.9 ±2.8	1.8 ±0.6	1.6 ±0.1
	On Ice	2.3 ±0.5	1.8 ±0.5	1.7 ±0.5	2.2 ±0.4	2.0 ±0.7
	In Ice	1.8 ±0.3	2.2 ±0.3	2.0 ±0.7	2.1 ±0.7	1.6 ±0.7
Clove Oil	Fresh	1.8 ±0.5	1.7 ±0.5	2.3 ±1.0	1.7 ±0.6	2.3 ±0.4
	On Ice	3.0 ±0.5	2.2 ±0.3	1.7 ±0.3	2.2 ±0.1	2.0 ±0.8
	In Ice	1.9 ±0.4	2.4 ±0.6	1.5 ±0.3	2.0 ±0.6	2.1 ±0.8
MS-222	Fresh	1.8 ±0.7	1.9 ±0.3	2.0 ±0.4	2.2 ±0.6	1.6 ±0.3
	On Ice	1.9 ±0.2	2.0 ±0.4	1.8 ±0.5	2.6 ±0.7	2.2 ±0.9
	In Ice	2.0 ±0.4	2.8 ±0.1	1.9 ±0.1	2.1 ±0.8	1.9 ±0.8
Control	None	2.1 ±0.5	-	-	-	-

Rb (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184*	365
Direct Cull	Fresh	0.063 ±0.029	0.070 ±0.015	0.049 ±0.013	-	0.059 ±0.016
	On Ice	0.056 ±0.026	0.045 ±0.013	0.063 ±0.021	-	0.044 ±0.022
	In Ice	0.062 ±0.018	0.043 ±0.020	0.074 ±0.030	-	0.059 ±0.008
2-phenoxyethanol	Fresh	0.073 ±0.017	0.049 ±0.014	0.053 ±0.011	-	0.063 ±0.021
	On Ice	0.050 ±0.012	0.054 ±0.014	0.029 ±0.014	-	0.054 ±0.014
	In Ice	0.052 ±0.029	0.065 ±0.018	0.048 ±0.017	-	0.044 ±0.012
Clove Oil	Fresh	0.054 ±0.012	0.043 ±0.014	0.051 ±0.029	-	0.060 ±0.015
	On Ice	0.052 ±0.024	0.055 ±0.025	0.063 ±0.020	-	0.056 ±0.008
	In Ice	0.044 ±0.012	0.058 ±0.021	0.057 ±0.023	-	0.065 ±0.019
MS-222	Fresh	0.045 ±0.021	0.066 ±0.044	0.044 ±0.020	-	0.054 ±0.013
	On Ice	0.064 ±0.009	0.064 ±0.025	0.063 ±0.021	-	0.067 ±0.013
	In Ice	0.043 ±0.017	0.047 ±0.009	0.046 ±0.013	-	0.065 ±0.022
Control	None	0.035 ±0.022	-	-	-	-

Sr (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184	365
Direct Cull	Fresh	1180.1 ±151.4	1192.3 ±88.8	1256.3 ±205.4	1357.4 ±135.2	1211.0 ±669.1
	On Ice	1380.1 ±310.4	1208.0 ±72.4	1057.2 ±313.4	1384.6 ±113.7	1326.8 ±166.8
	In Ice	1413.1 ±122.7	1040.4 ±347.2	1180.6 ±340.5	1396.9 ±87.3	1405.6 ±285.3
2-phenoxyethanol	Fresh	1169.5 ±215.6	1271.7 ±180.7	1130.5 ±207.2	1349.2 ±75.1	1431.9 ±240.5
	On Ice	1254.4 ±212.2	1302.8 ±158.0	1227.3 ±275.2	1168.6 ±240.0	1305.2 ±356.1
	In Ice	1097.9 ±160.5	1268.3 ±90.8	1149.0 ±180.3	1408.4 ±133.7	997.2 ±488.9
Clove Oil	Fresh	1460.4 ±135.5	1157.2 ±146.4	1088.0 ±244.5	1110.1 ±500.9	1291.5 ±423.0
	On Ice	1392.9 ±118.4	1332.3 ±113.8	1182.2 ±170.1	1141.0 ±472.3	1202.6 ±230.8
	In Ice	1159.8 ±372.5	1416.2 ±38.2	1202.1 ±88.2	1168.0 ±323.5	1123.9 ±744.4
MS-222	Fresh	1201.1 ±325.1	1350.8 ±214.8	1325.9 ±153.9	1339.8 ±74.4	1284.3 ±150.8
	On Ice	1072.2 ±461.0	1281.0 ±227.4	1224.7 ±105.8	1314.3 ±118.1	1641.1 ±438.6
	In Ice	1228.4 ±205.6	1142.2 ±342.6	1268.0 ±289.2	1269.1 ±60.4	1425.4 ±475.0
Control	None	1239.0 ±162.2	-	-	-	-

Table 2.4. Continued.

Ba (mg/kg)	Transport Method	Storage Duration (Days)				
		1	5	25	184	365
Direct Cull	Fresh	1.43 ±0.23	1.36 ±0.18	1.64 ±0.38	1.59 ±0.30	1.82 ±0.81
	On Ice	1.58 ±0.37	1.62 ±0.22	1.33 ±0.42	1.60 ±0.33	1.83 ±0.36
	In Ice	1.62 ±0.23	1.25 ±0.43	1.14 ±0.61	1.75 ±0.31	1.92 ±0.25
2-phenoxyethanol	Fresh	1.47 ±0.36	1.54 ±0.31	1.09 ±0.55	1.64 ±0.17	1.86 ±0.36
	On Ice	1.39 ±0.54	1.53 ±0.12	1.50 ±0.26	1.31 ±0.21	1.84 ±0.34
	In Ice	10.8 ±0.44	1.54 ±0.22	1.60 ±0.24	1.64 ±0.13	1.50 ±0.60
Clove Oil	Fresh	1.75 ±0.13	1.33 ±0.23	1.14 ±0.42	1.29 ±0.59	1.92 ±0.55
	On Ice	1.59 ±0.25	1.68 ±0.24	1.43 ±0.26	1.41 ±0.61	1.63 ±0.31
	In Ice	1.43 ±0.51	1.67 ±0.27	1.34 ±0.15	1.41 ±0.46	1.66 ±1.05
MS-222	Fresh	1.62 ±0.33	1.71 ±0.25	1.41 ±0.38	1.50 ±0.26	1.74 ±0.32
	On Ice	1.26 ±0.58	1.51 ±0.29	1.52 ±0.12	1.61 ±0.17	2.18 ±0.60
	In Ice	1.09 ±0.57	1.36 ±0.32	1.54 ±0.42	1.54 ±0.17	1.96 ±0.54
Control	None	1.62 ±0.31	-	-	-	-

* Note: Samples for Rb were found to be below the LOD for the 6 month (184 days) data and were omitted.

Mean otolith elemental concentrations showed some variability between one or more of the storage periods and the methods of dispatch and anaesthesia (Figure 2.1.). Most notable were the storage periods of 1 and 365 days for the element Mg, and 1, 25 and 365 days for Mn. However, conversely, one period of storage (184 days) showed very little variability for the elemental concentrations of Mg. Rb indicated variability for the mean elemental concentrations for the freezer storage periods of 1, 5 and 25 days, however, the period of 365 showed little variability across the mean values for each of the transport and anaesthesia methods used (see Figure 2.1.).

Mean elemental concentrations of Na were high for the storage period of 1 day and the anaesthetic MS222 when transported on ice when compared to the remaining concentrations (Table 2.4.). Similarly, mean concentrations were high for Mg and 184 days storage. However, Mg concentrations remained relatively consistent for the remaining periods of storage. Similar results were observed for the elements Sr and Ba.

2.5.3. Whole otolith analysis using the control otoliths

MANOVA, conducted on the element: Ca ratio Log_{10} transformed elements Na, Mg, K, Mn, Sr and Ba indicated no significant differences in the elemental concentrations of the six elements between the three methods of transportation and the control otoliths (Wilks' Criterion: $F_{18, 846} = 0.892$, $P = 0.588$), similarly, no significant differences were observed between each of the four anaesthetic protocols and the control (Wilks' Criterion: $F_{24, 1040} = 0.930$, $P = 0.560$). However, significant differences were observed between one or more of the six elements when looking at storage duration and the control (Wilks' Criterion: $F_{30, 1190} = 6.648$, $P < 0.001$). Individual ANOVA's run in conjunction with the MANOVA indicated Mg, K, and Mn were significantly different in their elemental concentrations between one or more of the storage periods and the control (Mg: $F_{5, 307} = 7.95$, $P < 0.001$; K: $F_{5, 307} = 3.68$, $P < 0.003$; Mn: $F_{5, 307} = 2.72$, $P < 0.020$).

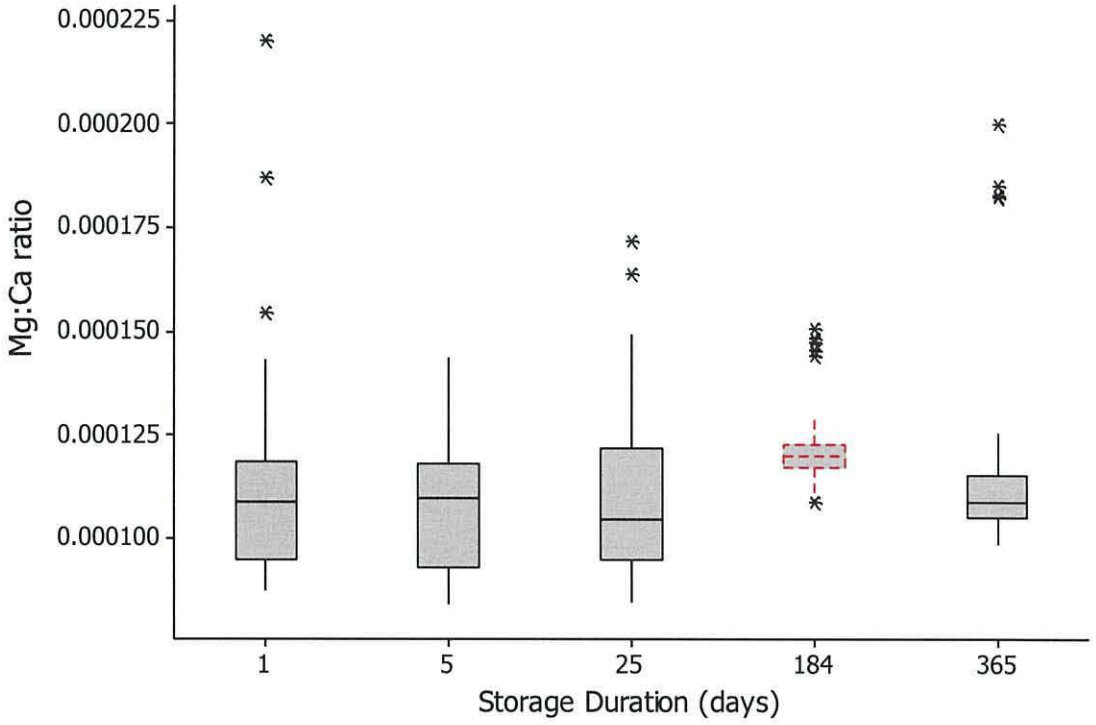
Post-hoc analysis indicated those differences were between the 184 day storage period and the storage periods 1, 5 and 25 days and the control (Bonferroni Test $P < 0.05$) for the element Mg. Similarly, significant differences were also observed between the storage period of 184 days and the storage period of 5 days for K (Bonferroni Test $P < 0.05$). Mn however, indicated significant differences in elemental concentrations between the storage period of 1 day and 365 days (Bonferroni Test $P < 0.05$). Due to unbalanced nesting (retaining only 10 otolith samples for the control), testing the interactions of all the methods deployed for this experiment and the control samples using the 3-Way ANOVAs were not possible and the control otoliths were removed from any further analyses.

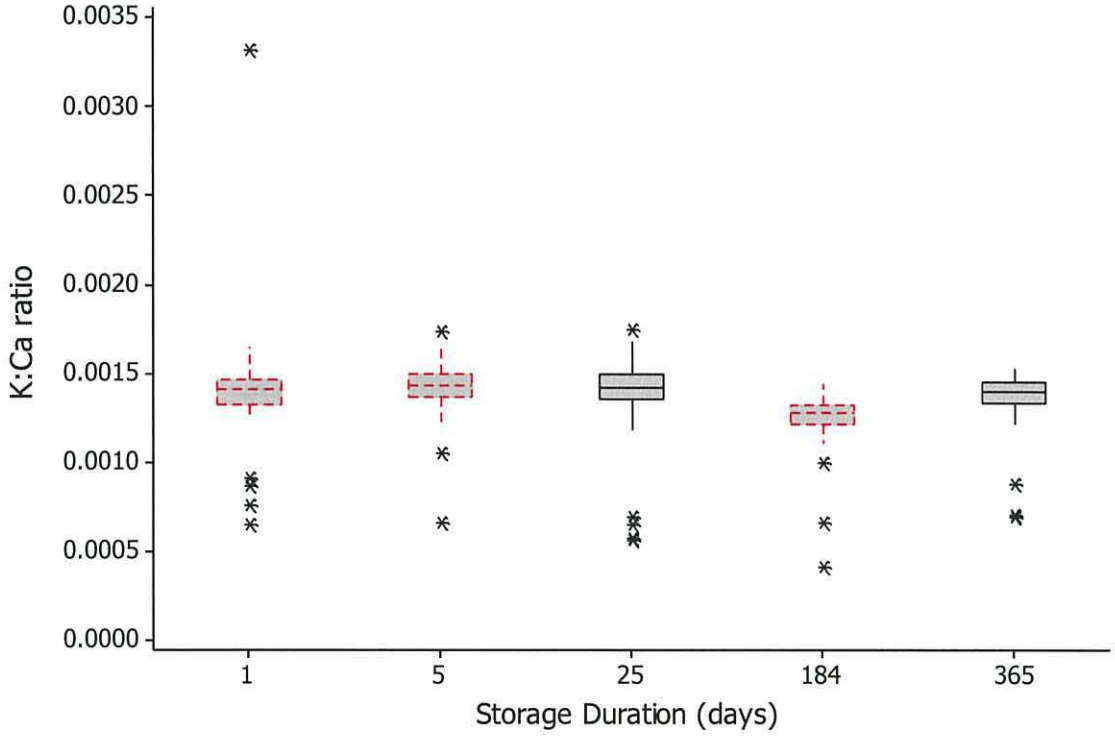
2.5.4. Whole otolith analysis and their interactions between methods

MANOVA conducted on the six elements Na, Mg, K, Mn, Sr and Ba indicated no significant differences in the elemental concentrations when looking at transportation method (Using Wilks' Criterion: MANOVA: $F_{12, 580} = 0.749$, $P = 0.703$), Similarly, no significant elemental differences were observed for the same six elements and the four methods of anaesthesia (Using Wilks' Criterion: MANOVA: $F_{18, 817} = 0.850$, $P = 0.640$). However, as observed for the analysis using the control, significant differences were observed between the elements Na, Mg, K, Mn, Sr and Ba and storage duration (using Wilks' Criterion: MANOVA: $F_{24, 1005} = 7.918$, $P < 0.001$).

Analysis of the four methods of dispatch, three transportation methods and the five methods of storage combined with the interactions between each of the main methods was conducted using a 3-Way ANOVA separately on the Log_{10} data for Na, Mg, K, Mn, Sr and Ba. Results for three of the elements (Na, Sr and Ba) showed no significant effect of dispatch, transportation or storage duration or interactions between each of the three main methods used (Table 2.5.). However, results for the 3-way ANOVAs indicated a significant effect of storage duration when looking at Log_{10} elemental data for Mg, K and Mn. *Post-hoc* analysis (using a Bonferroni test) indicated similar results to those observed when comparing the same elements with the control otoliths, with significant differences observed for Mg, indicating elemental concentrations differing between the storage period of 184 days and the storage periods of 1, 5 and 25 days (all $P < 0.05$). Similarly, significant differences were observed between the storage duration of 365 days and the storage period of 1 day for Mn ($P < 0.05$). However, when measuring the significant differences in the elemental concentrations of K between storage periods, significant differences were observed between the storage period of 184 days and the storage periods of 1 and 5 days, the addition of the storage period of 1 day was observed to be significant (both $P < 0.05$).

Using boxplots (Figure 2.2.), the data distribution underlying the significant differences in the elemental concentration of Mg, K and Mn between storage times, detected using the *post-hoc* analysis (Bonferroni test) are presented. Most notable are the small 25^{th} and 75^{th} percentiles observed for the median and the storage period of 6 months (184 days) for Mg. Both K and Mn indicate similar median values between freezer storage periods, with one notable outlier indicated for storage period of 1 day and the element K (see Figure 2.2.).





(Figure 2.2. and caption are continued overleaf)

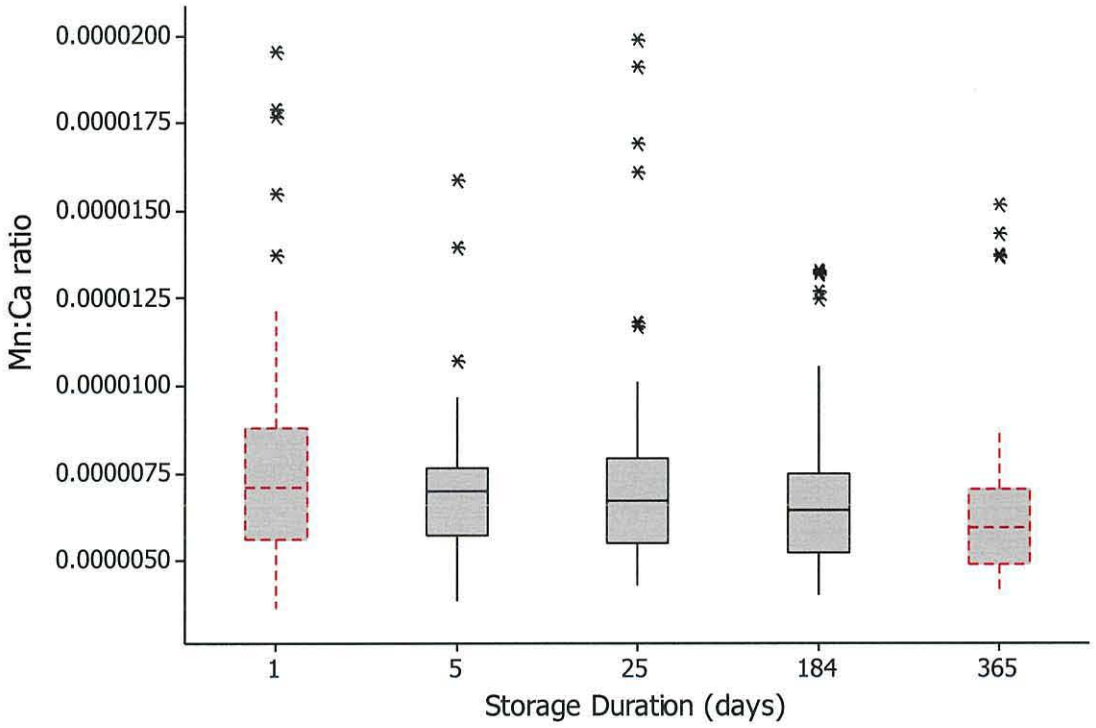


Figure 2.2. Boxplots showing the effects of storage period (n = 60 / storage period) on element: Ca ratios of Mg, K and Mn in juvenile sea bass otoliths. Significant differences between storage periods ($P < 0.05$) are highlighted red. Data are represented as median (horizontal lines), 25th and 75th percentile (bottom and top of box respectively), 5th and 95th percentiles are represented by the whiskers. Outliers indicating a value greater than the 95th percentile are indicated by *.

Table 2.5. 3-way ANOVAs for each of the Log_{10} elements (Na, Mg, K, Mn, Sr and Ba) in the otoliths of juvenile sea bass (*D. labrax*) and their interactions for the four anaesthetic methods, three methods of transportation and five periods of storage. Significance at the α ($P < 0.05$) level are indicated in **bold**.

Log_{10} Na	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Transport method	2	0.01105	0.01023	0.00512	0.83	0.436
Storage Time (Days)	4	0.03317	0.03387	0.00847	1.38	0.242
Anaesthetic Method	3	0.02236	0.02413	0.00805	1.31	0.272
T- Method * Storage Time	8	0.05451	0.05538	0.00692	1.13	0.346
T-Method * Anaesthetic Method	6	0.01254	0.01438	0.00240	0.39	0.885
Storage Time * Anaesthetic Method	12	0.04177	0.04296	0.00358	0.58	0.856
T- Method * Store Time * Anaesthetic Method	24	0.14208	0.14208	0.00592	0.96	0.517
Error	238	1.46334	1.46334			
Total	297	1.78080				
R-Sq = 17.83%						

Log_{10} Mg	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Transport method	2	0.00346	0.00424	0.00212	0.52	0.597
Storage Time (Days)	4	0.12730	0.12624	0.03156	7.71	**<0.001**
Anaesthetic Method	3	0.00898	0.00802	0.00267	0.65	0.582
T- Method * Storage Time	8	0.02410	0.02190	0.00274	0.67	0.719
T-Method * Anaesthetic Method	6	0.00520	0.00551	0.00092	0.22	0.969
Storage Time * Anaesthetic Method	12	0.04713	0.04780	0.00398	0.97	0.475
T- Method * Store Time * Anaesthetic Method	24	0.07739	0.07739	0.00322	0.79	0.751
Error	238	0.97440	0.97440	0.00409		
Total	297	1.26795				
R-Sq = 23.15%						

Table 2.5. Continued.

Log_{10} K	DF	Seq SS	Adj SS	Adj MS	F	P
Transport method	2	0.01384	0.01253	0.00627	0.91	0.405
Storage Time (Days)	4	0.11223	0.11224	0.02806	4.06	**0.003**
Anaesthetic Method	3	0.02125	0.02359	0.00786	1.14	0.334
T- Method * Storage Time	8	0.06462	0.06495	0.00812	1.18	0.315
T-Method * Anaesthetic Method	6	0.00893	0.01002	0.00167	0.24	0.962
Storage Time * Anaesthetic Method	12	0.05897	0.06135	0.00511	0.74	0.711
T- Method * Store Time * Anaesthetic Method	24	0.01474	0.01474	0.00614	0.89	0.617
Error	238	1.64378	1.64378	0.00691		
Total	297	2.07102				
R-Sq = 20.63%						

Log_{10} Mn	DF	Seq SS	Adj SS	Adj MS	F	P
Transport method	2	0.00752	0.01216	0.00608	0.34	0.715
Storage Time (Days)	4	0.24990	0.25631	0.06408	3.53	**0.008**
Anaesthetic Method	3	0.01553	0.01518	0.00506	0.29	0.841
T- Method * Storage Time	8	0.27557	0.27451	0.03431	1.89	0.062
T-Method * Anaesthetic Method	6	0.04543	0.04525	0.0754	0.42	0.868
Storage Time * Anaesthetic Method	12	0.26971	0.27330	0.02277	1.26	0.246
T- Method * Store Time * Anaesthetic Method	24	0.46808	0.46808	0.01950	1.08	0.373
Error	238	4.31684	4.31684	0.01814		
Total	297	5.64859				
R-Sq = 23.58%						

Table 2.5. Continued.

Log_{10} Sr	DF	Seq SS	Adj SS	Adj MS	F	P
Transport method	2	0.01068	0.00931	0.00466	0.45	0.638
Storage Time (Days)	4	0.05758	0.05372	0.01343	1.30	0.271
Anaesthetic Method	3	0.04253	0.04508	0.01503	1.45	0.228
T- Method * Storage Time	8	0.07762	0.0880	0.00985	0.95	0.473
T-Method * Anaesthetic Method	6	0.02098	0.02138	0.00356	0.34	0.912
Storage Time * Anaesthetic Method	12	0.07413	0.07487	0.00624	0.60	0.838
T- Method * Store Time * Anaesthetic Method	24	0.20025	0.20025	0.00834	0.81	0.726
Error	238	2.45880	2.45880	0.01033		
Total	297	2.94257				
R-Sq = 16.44%						

Log_{10} Ba	DF	Seq SS	Adj SS	Adj MS	F	P
Transport method	2	0.03832	0.03572	0.01786	0.92	0.402
Storage Time (Days)	4	0.15267	0.15198	0.03800	1.95	0.103
Anaesthetic Method	3	0.08020	0.08205	0.02735	1.40	0.243
T- Method * Storage Time	8	0.15105	0.14969	0.01871	0.96	0.468
T-Method * Anaesthetic Method	6	0.06451	0.06075	0.01013	0.52	0.793
Storage Time * Anaesthetic Method	12	0.10876	0.10618	0.00885	0.45	0.939
T- Method * Store Time * Anaesthetic Method	24	0.47129	0.47129	0.01964	1.01	0.458
Error	238	4.64081	4.64081	0.01950		
Total	297	5.70761				
R-Sq = 18.69%						

2.6. Discussion

When measuring the microchemistry of fish otoliths using inductively-coupled plasma mass spectrometry (ICP-MS) there are a number of confounding factors that need to be dealt with in order to eliminate bias in the data obtained. The problems of possible contamination during the extraction and preparation of fish otoliths have been highlighted (*e.g.* Thresher, 1999; Rooker *et al.*, 2001; Swan *et al.*, 2006) and how to eliminate this suggested (see Campana, 1999). Furthermore, the potential for errors at the analytical stage have also been identified (Thresher, 1999). In his influential review, Campana (1999) outlined strict protocols for decontaminating equipment prior to use using various acid-washing techniques and concentrations methods that can be used to reduce / remove these sources of error. There is now an extensive literature on acid-washing protocols for use in in fish otolith microchemistry: cleaned in dilute HNO_3 (Rooker *et al.*, 2001), washed in 10% HNO_3 (Brophy *et al.*, 2003), soaked for 24 hours in 13% HNO_3 (Hedges *et al.*, 2004), leached in 3N HCl and 3N HNO_3 for 7 days (Brown, 2006). The use of non-metallic dissecting equipment can also eliminate contamination during the otolith extraction phase (*e.g.* Thorrold *et al.*, 1997; Brophy *et al.*, 2004; Hedges *et al.*, 2004). In addition, it has been suggested that the work environment is also decontaminated to eliminate the problems of contamination occurring while preparing fish otoliths for analysis after extraction (Campana, 1999; Rooker *et al.*, 2001).

In the present study, cleansing protocols recommended by the B.G.S. were strictly adhered too during the extraction, laboratory processing and analytical phases of the study. Therefore, it is assumed that by observing these protocols any variability observed in the data set collected will not be due to contamination during preparation / analysis but will be due to the experimental treatments. The main aim of this study was to examine whether different pre-analytical protocols, *i.e.* the method of euthanasia after capture, the method of transportation and whether short- and long-term freezer storage may affect otolith elemental concentrations measured using ICP-MS. The methods of euthanasia and transportation and the storage times selected were chosen to represent some of the most common methods that could be deployed by researchers, scientific and government institutions in microchemistry studies.

Elemental concentrations between otolith pairs extracted from the same fish for the control indicated no significant changes between both the right and left sagittal otolith

(see Proctor and Thresher, 1998; Swan *et al.*, 2006). However, variations were observed between freezer storage duration and Mg, K and Mn and it is possible that this was caused by the presence of crystalline otoliths in some of the otoliths analysed. Crystalline otoliths are natural variations in otolith structure, caused by higher proportions of vaterite (or the rarer form, calcite), which give the affected sagittal otolith a glass-like appearance when compared to a none affected aragonite otolith (Gauldie, 1993; Swan *et al.*, 2006). The replacement of aragonite by vaterite can influence the uptake of certain elements *e.g.* Mg, Mn and Sr into the otolith (Gauldie, 1996; Brown and Severin, 1999; Tomás and Geffen, 2003). It is possible that this may be the cause of the significant differences observed in the present study, however, all otoliths used in the present study were checked under the microscope and no crystalline otoliths were used in the present study.

It is recognized that some elements are more labile and are more easily leached from the otolith matrix and therefore may be more prone to alteration within the lattice structure of the otolith Hedges *et al.* (2004). For example, elements such as Na, S and K which do not readily substitute for Ca within the $CaCO_3$ otolith matrix and are more physiologically regulated may be influenced by external factors such storage method and storage time. However, elements which have a similar ionic radii and ionic charge which match that of the free Ca^{2+} cation (*e.g.* the elements Mn, Rb, Sr, and Ba) are thought to be more tightly bound within the $CaCO_3$ otolith matrix and not as susceptible (Campana, 1999; Hedges *et al.*, 2004). In the present study, there were minimal effects of methods of dispatch and transportation and storage duration on the otolith chemistry of juvenile sea bass. The only significant effects observed were for storage time, specifically 6 months storage for Mg and K and comparing short-term (1 day) and long-term (1 year) storage for Mn.

Mg (a Group II alkaline-earth metal) has been described by some authors as being more resilient to the effects of storage and handling / cleaning protocols (see Rooker *et al.*, 2001; Swan *et al.*, 2006) as it is assumed to substitute for the element Ca within the crystal lattice structure of the otolith. Therefore it is thought to be more tightly-bound within the otolith matrix (Proctor and Thresher, 1998; Rooker *et al.*, 2001) compared to those elements (such as C,H, O, N, P, and S) which are associated with the organic matrix (Dove *et al.*, 1996; Campana, 1999; de Pontual and Geffen, 2002). However, conversely Milton and Chenery (1998) reported a small but significant effect of storage time on otolith Mg concentrations using LA-ICP-MS and concluded that otoliths removed from the fish shortly after capture and then stored dry had the least variation on the Mg

elemental concentrations. In the present study, the significant differences observed for Mg in the otoliths after 6 months storage are considered to be biologically meaningless (*i.e.* a chance Type 1 error) since the data for 1, 5 and 25 days and for 12 months were all similar. The random nature of sampling otoliths from the freezer over time to assess the effects of storage duration, together with the reduced variance in Mg concentration for the 6 month storage samples (see Figure 2.1.), are thought to explain the significant result obtained at 6 months.

The present study was conducted because some studies had suggested a change in the elemental composition of otoliths, including Mg, when stored frozen for differing periods of time (*e.g.* Na, Mg, S, Cr, Zn and Ba: Milton and Chenery, 1998; Proctor and Thresher, 1998; Rooker *et al.*, 2001; Brophy *et al.*, 2003; Swan *et al.*, 2006). The results of the present study do not support this contention, however, the analysis by Milton and Chenery (1998) involved the use of LA-ICP-MS, compared to sb-ICP-MS used in the present study. It is possible that the analysis of a very localized region of the otolith using LA-ICP-MS, compared to the whole-otolith approach adopted in the present study may explain the differences observed.

Where temporal changes in otolith element concentrations have been observed, the trends in the data are not consistent. For example, Milton and Chenery (1998) indicated concentrations of Na and Mg were higher in the otoliths of one year old Terubok shad *Tenualosa toli* extracted after storage frozen for one day compared to longer time periods. In contrast, increased concentrations of Mg and Zn were observed in the otoliths from juvenile Atlantic herring *Clupea harengus* with extended freezing periods although this may be attributable to *post mortem* contamination (Brophy *et al.*, 2003). Finally, Rooker *et al.* (2001) found no significant effects of freezing for seven days on the elemental concentrations of Mg in the blackfin tuna *Thunnus atlanticus* but they observed increased Mg and decreased Na concentrations in the otoliths from whole fish samples stored on ice for a three day period. Thus the results for the effects of storage on Mg are inconsistent and may in part be due to the methodology employed (LA- vs. sb-ICP-MS) and possible contamination during the storage, extraction and preparatory phases prior to analysis.

A study by Gauldie *et al*, (1998) has shown that there is considerable potential for the movement of large molecules by fluid migration through the protein – aragonite matrix of the otolith. As a result of stress induced during capture, changes in alkalinity and the composition of the endolymphatic fluid that surrounds and bathes the otoliths (Mugiya and Uchimura, 1989; Kalish, 1991, 1992; Payan *et al.*, 1998) may occur. Indeed,

Swan *et al.* (2006) commented on the possible effects of delayed extraction when sampling fish species which inhabit deep sea waters due to the length of time required bringing those fish samples to the surface. There is also some evidence that the otolith concentration of some elements (*e.g.* Group I elements such as Li, Na and K) may be affected by *post mortem* handling effects causing the degradation and biological breakdown of the surrounding tissue or during the slow freezing process itself (Proctor and Thresher, 1998; Rooker *et al.*, 2001; Brophy *et al.*, 2003; Swan *et al.*, 2006). Since fluid migration is known to occur within aragonite structures (see Gauldie *et al.*, 1998) this may be one pathway by which the more labile and easily affected elemental concentrations of certain Group I elements are changed (Swan *et al.*, 2006). It is possible that Mg might be similarly affected. Thus, there may be some transport of elements in / out of the otolith during the freezing and thawing process and if the same otoliths are also used for ageing there could be elemental uptake or leaching from the surface of the otolith if they are immersed in clarifying liquids for viewing under the light microscope for aging analysis (Proctor and Thresher, 1998). In addition, residue on the surface of the otoliths not removed by robust cleaning may also influence the measurements obtained, for example for Mg (see Brophy *et al.*, 2003). However, the results of the present study would suggest that where strict protocols are implemented and whole otolith analysis is conducted, changes in magnesium over time are not significant for storage periods of up to a year.

In the present study, a significant difference was observed in otolith K concentration between 1 / 5 days and 6 months storage. Alkali metals such as K (and similar elements *e.g.* Li, Na, and Rb and the halogens Cl and Br) are reported to be extremely labile within the otolith crystal lattice structure and are therefore prone to possible alterations (Milton and Chenery, 1998; Proctor and Thresher, 1998; Rooker *et al.*, 2001; Hedges *et al.*, 2004). The fact that these more labile elements are present within the $CaCO_3$ otolith matrix as single-valent inclusions makes these elements less useful as chemical markers of population origin in fish stock identification or movement tracking studies (Campana, 1999; Rooker *et al.*, 2001). The lability of elements K (and other elements) has been well documented when studying other biological structures comprising of aragonite (*e.g.* corals, Proctor and Thresher, 1998). The apparent lability of K may help to explain the reduced concentrations observed in the present study after 6 months storage but the decline did not continue with 12 months storage. This observation,

together with the reduced variance in the 6 month K data set (see Figure 2.1. and Table 2.4.), again suggest a chance Type 1 error

One possible explanation for the significant results observed between storage periods for Mg and K could be due to the effect of day-to-day machine variability since samples were run on the ICP-MS on separate days. Given the experimental design and machine availability at the B.G.S. it was not possible to run all the samples on the same analytical run on the machine. However, the use of quality control standards (within the ICP-MS) and the further use of external calibration multi-elemental standard techniques would have reduced the possibility of machine inter-run variability (reproducibility) for all the samples run during the present study.

In the present study, Mn was significantly lower after 12 months freezer storage compared to one day. The processes of incorporation of some elements such as Li, Mn, Co and Ba, are poorly understood and maybe due to physiological functions (Milton and Chenery, 1998). However, the effects of storage on the elemental concentrations of Mn using a preserving medium such as ethanol indicated Mn was found not to be susceptible to any changes (*e.g.* leaching). This may be part due to the element substituting for Ca in $CaCO_3$ within the otolith matrix (being a $^{+}2$ cation) within geological environments (Speer, 1983; Hedges *et al.*, 2004). Similarly, Milton and Chenery (1998) also commented upon the lack of storage effects on Mn in fish otoliths. However, since Mn can be one of the divalent ions most commonly used to discriminate between populations / sources of fish (*e.g.* Wells *et al.*, 2000; Walther and Thorrold, 2008; Walther *et al.*, 2008; Ramsay *et al.*, 2011, 2012; see review by Gillanders and Kingsford, 2000) and the results of this study have suggested that long-term storage in frozen fish carcasses may reduce Mn concentration in the otolith, it is advised that otoliths are removed for analysis before the period of one year storage is reached.

The last remaining two elements assayed for in the present study (Sr and Ba) are thought to be more robust since as divalent ions they will substitute for Ca within the crystal lattice of the otolith matrix (Speer, 1983; Kalish, 1989; Rooker *et al.*, 2001). Sr has also been found to be stable within other calcareous structures, such as coral aragonite (Amiel *et al.*, 1973). The results of the present study support this assumption since methods of dispatch and transportation, dispatch and storage durations of frozen whole fish for up to one year did not affect the Sr and Ba concentrations in the otoliths. This is an important result as Sr and Ba are the two main divalent ions used in fish otolith microchemistry studies to discriminate between populations / sources of fish (*e.g.*

Gillanders, 2005; Wells *et al.*, 2000; Walther and Thorrold, 2008; Walther *et al.*, 2008; Ramsay *et al.*, 2011, 2012).

2.6.1. Conclusions

The use of the trace element composition of fish otoliths as a method of studying the population origin and movement patterns of fish species has grown in the last 20 years (Thresher, 1999; Gillanders, 2002; Elsdon *et al.*, 2008; Sturrock *et al.*, 2012; Walther and Limburg, 2012). However, this use is not underpinned by a comprehensive knowledge of the factors determining the uptake and retention of trace elements within the otolith as it grows. The processes which define the method of elemental incorporation and the sequential addition of protein layers within the $CaCO_3$ (primarily aragonite: see Degens, *et al.*, 1969; Campana and Neilson, 1985; Campana, 1999) lattice structure of the otolith and their molecular structure are complex and have only been described in some detail by a few authors (*e.g.* Degens *et al.*, 1969; Kalish, 1991; Campana, 1999; Thresher, 1999). These biological and physical processes involve the uptake of fluids and organic molecules from the ambient water mass in which the fish is residing, with those fluids then passing through the fish's gills into the blood plasma and then finally into the endolymphatic fluid crossing yet another membrane (Bath *et al.*, 2000; see review by Campana, 1999). Clearly, the movement of elements along this multi-compartmental pathway could be influenced by many factors and so could be affected by *post mortem* handling procedures and stresses (Rooker *et al.*, 2001). This may affect the concentrations of elements measured in the otolith itself and influence the conclusions reached in identifying fish to their natal nursery ground and studying possible movement patterns.

Previous worker, such as Milton and Chenery (1998) and Proctor and Thresher (1998), have suggested that the storage and the subsequent handling procedures of otoliths can influence their chemical composition. Furthermore, Campana (1999) and Rooker *et al.* (2001) have also suggested analytical handling procedures may also affect the measurements of those otolith samples. Elements which are more labile and which are not tightly bound to the $CaCO_3$ otolith matrix, *e.g.* the elements Na, K, Mg and S, are thought to be prone to alteration, whereas those elements more tightly bound to the otolith matrix, *e.g.* Sr and Ba, are not (Milton and Chenery, 1998; Proctor and Thresher,

1998; Hedges *et al.*, 2004). The results of the present study have indicated possible variations in the elemental concentrations of Mg, K and Mn by means of the most commonly used methods of euthanasia / transportation and storage duration in sea bass otoliths. Additionally the methods used above indicated no variation in the chemical concentrations of Na, Sr and Ba. Moreover, although the storage period of 6 months indicated changes in the elemental concentration of Mg and K, and differences in Mn were observed between short- and long-term storage (1 day vs. 12 months), taken together the evidence pointed to these results being a chance effect with the fish being analysed rather than a real significant variation in otolith chemistry.

Chapter 3

Temporal stability of a nursery-specific chemical tag in the otoliths of juvenile plaice (*Pleuronectes platessa* L.)

Abstract

Trace and minor-trace elements incorporated within the otoliths of fish have been used to identify population structure and identify life history patterns over spatial scales in both marine and freshwater habitats. However, water chemistry within these habitats typically varies over time. Therefore, if we are to be confident in our interpretation of fish movements over a spatial scale, it is particularly important to have some understanding of the possible temporal variation within those environments. Studies looking into temporal stability are few and have shown both inter-annual and intra-annual variability when measuring the chemistry of otoliths extracted from fish sampled from both rivers and estuaries and along coastal regions. Variations in the trace elemental concentrations have been observed over temporal scales varying from between 2-3 years to an extended time period of 4-13 years. In this study juvenile plaice (*Pleuronectes platessa*) were sampled from two different nursery grounds over a period of 7 years to assess if temporal stability on both an inter-annual (between years) and intra-annual (between both season and month) scale could be observed. In the present study, significant variations were observed in the elemental concentrations of Na, Mg, K, Sr and Ba in sagittal otoliths of juvenile plaice on both an inter-annual and intra-annual timescale at two nursery grounds at Llanddona and Llanfairfechan for the time period 2004-2010. Specifically, significant inter-annual (between years) variation in the measured trace elemental compositions of plaice otoliths were observed between each of the 3 years 2007-2010 for Mg, between each of the 5 years 2005-2010 for Sr and between the 2 years 2009-2010 for Na at Llanfairfechan. Similar results in elemental variation were observed for Na between each of the 4 years 2007-2010, between the 3 years 2007-2010 for K, and between the two years 2007-2009 and 2009-2010 for Sr and Ba respectively at Llanddona. Some degree of temporal stability however, could be observed in the elemental concentrations of Na, Mg and Ba at the nursery ground at Llanfairfechan and the elemental concentrations of Na, K, Sr and Ba within the site at Llandonna over short time scales (*i.e.* 2-3 year periods), increasing to 4 years (2004-2007) for Na and Ba at Llanfairfechan and K and Ba at Llanddona. The results of this study indicate that temporal stability on an inter-annual scale can be observed over the short term: *i.e.* 2-3 years, and for some elements (*e.g.* Ba), up to 4 years.

3.1. Introduction

The use of calcified structures, and in particular the use of otoliths (*e.g.* Limburg, 1995; Elsdon and Gillanders, 2006*b*), in microchemistry as a tool to identify stock structure and movement patterns and to reconstruct the environmental histories of various marine and freshwater fish species have been well reviewed (*e.g.* Thresher, 1999; Gillanders, 2002; Elsdon *et al.*, 2008; Sturrock *et al.*, 2012; Walther and Limburg, 2012). In addition, the use of these elemental signatures has become an invaluable tool for fisheries scientists (Campana, 1999) in understanding the connectivity of both local spawning grounds (Begg *et al.*, 1998; Edmonds *et al.*, 1999; Patterson *et al.*, 1999; Newman *et al.*, 2000), to identify juvenile nursery grounds (Thorrold *et al.*, 1998*a*; Spencer *et al.*, 2000) and to reconstruct patterns of residency of fish migrating between freshwater and marine environments (Kalish, 1990; Secor, 1992; Gillanders and Kingsford, 1996).

Differences in the ambient environmental conditions experienced by fish *e.g.* temperature, salinity and trace elements within the water (see Gillanders, 2005) are reflected by changes in elemental concentrations in the otoliths (Limburg, 1998; Limburg *et al.*, 2010). This has enabled scientists to distinguish between water masses that differ in their chemistry, for example between different streams, different estuaries, between estuaries and coastal waters and between inshore and offshore reef habitats (Gillanders and Kingsford, 1996; Thorrold *et al.*, 1998*b*; Gillanders and Kingsford, 2000; Ramsay *et al.*, 2011). In addition, the use of the juvenile portion or "nucleus" (as defined by Campana and Neilson, 1985) of the adult otolith has the potential to enable the classification of adult fish back to their nursery grounds (Campana *et al.*, 1994; Gillanders, 2002; see Chapter 4). However, for adult fish to be retrospectively identified back to their natal habitats using the nucleus, either trace elemental signatures in the environment which were incorporated within the otoliths would have to remain constant over time (Elsdon and Gillanders, 2006*b*; Tanner *et al.*, 2012), or a long-term baseline for those natal habitats would need to be established by sampling each cohort of fish (Gillanders, 2002).

Elsdon and Gillanders (2006*b*) have suggested that if elemental concentrations are highly dynamic and / or vary considerably on small temporal scales within a given water body / environment (*e.g.* within estuarine environments), then fish will need to occupy that environment for a sufficient period of time to allow the incorporation of those

elements to be detected accurately in the otolith matrix; for example, in excess of 20 days has been cited by Elsdon and Gillanders (2005). There is evidence to suggest that the elemental signatures in the otoliths of juvenile fish residing in different estuaries may vary between years (*i.e.* inter-annual variability) (Gillanders and Kingsford, 2000), with the potential for variability to also be observed on a shorter temporal scale within year (*i.e.* intra-annual variability) (Gillanders, 2002). Indeed, if the chemical concentration incorporated within calcified structures varies over short time periods, they would represent what would be construed as a mixed signal for that environment / habitat and would therefore provide extremely poor classification results (Elsdon and Gillanders, 2006*b*). Therefore, if the elemental composition of otoliths are to be used as reliable environmental markers of origin and allow populations of fish to be correctly classified, then those results must be reproducible and be either consistent over time or (if they are not) exhibit variability on a temporal scale, matched plausibly to noticeable environmental or stock variations (Thresher, 1999).

The understanding of any temporal changes in elemental concentrations and their uptake into otoliths is fundamental if we are to use this technique to address ecological questions (Elsdon and Gillanders, 2006*b*). However, few studies so far have attempted to assess the changes in the chemical composition of specific elements over large temporal scales. This is worrying since the use of otolith microchemistry in fish biology is increasing rapidly with no fewer than 700 peer reviewed papers published between 1990 and 2010 (see review by Sturrock *et al.*, 2012) increasing to nearly 800 by the time of writing (797 Web of Science search 10-10-2013, topic: otolith chemistry Or otolith microchemistry). Furthermore, there has been a threefold increase in the publications using otolith chemistry to determine the movement patterns of fish populations from over 200 (see review by Elsdon *et al.*, 2008) increasing to nearly 600 (563 Web of Science search 10-10-2013); with around 35 of those publications investigating spatial variations observed in the elemental concentrations of otoliths (see reviews by Gillanders, 2002; Elsdon *et al.*, 2008). However, in contrast to these studies, fewer than 20 published studies have looked at temporal differences in otolith chemistry (Gillanders, 2002; Elsdon *et al.*, 2008; 17 Web of Science search 10-10-2013).

Microchemistry studies looking at spatial variability in otolith chemistry have tended to focus on transoceanic migrations or the detection of anadromy in fish and their movements between fresh and saltwater environments (Kalish, 1990), with few studies investigating the changes observed in fish migrating between estuarine and marine waters

(Gillanders, 2005). Similarly, studies looking at temporal stability have tended to focus more on estuarine environments (see Table 1. in Elsdon *et al.*, 2008), which by their very nature are highly dynamic with variability in their water chemistry affected by factors such as freshwater flow, local geological formations, turbidity, saltwater influx (Elsdon and Gillanders, 2003) and anthropogenic activities (*e.g.* land runoff; Li and Chan, 1979). There are very few studies investigating temporal variability in otolith chemistry compared to the number of studies examining spatial variability (Gillanders, 2002). However, the majority of published studies looking into temporal stability have tended to sample over a limited time period, usually ≤ 2 years (Edmonds *et al.*, 1992; Patterson *et al.*, 1999; Campana *et al.*, 2000) although some studies have worked with longer time series data, but not a continuous data set (*e.g.* Campana *et al.*, 2000) .

Furthermore, when analyzing the variation in otolith chemistry over a given geographical area, a knowledge of the level of variation in those chemical signatures over time within that area is crucial if we are to state that sampling at one period of time in one area is representative of sampling in the same area at a later period (Stransky *et al.*, 2005). Therefore, to use and assign these chemical signatures as a "tag" in identifying adults to their nursery ground of origin requires the chemical signal for that nursery ground to remain stable over time and distinctive relative to other regional nursery grounds (*i.e.* the inter-annual variability observed within a site being less than the variability observed between sites; Gillanders, 2002).

Juvenile European plaice (*Pleuronectes platessa*) were chosen for the study because (a) samples could be easily sourced from two recognized juvenile plaice nursery grounds within the local area (see Dunn and Pawson, 2002), (b) samples collected from 2004-2007 had already been retained and stored from a previous Ph.D. project studying juvenile plaice growth patterns from those two sites (Al-Rashada, 2009, unpublished data) and c) their early life stages are closely associated with the coastal environment. Furthermore their life history fits the criteria suggested by Elsdon and Gillanders (2005) in that their residency time within their chosen nursery area has been shown to be between 1 and 3 years (Bowers, 1963; Lockwood, 1974; Nash *et al.*, 1992; Nash *et al.*, 1994). In addition, previous work has shown that juvenile plaice exhibit site fidelity and homing behavior for their chosen nursery ground (Burrows *et al.*, 2004), with juveniles returning to their nursery of capture after being displaced 3.5 km offshore and when transplanted laterally (> 1 km) along the shore line (Riley (1973). The site fidelity, observed in juvenile plaice suggest that they are likely to experience the same physical

and biological conditions since settlement and this, combined with their natural homing trait (Burrows *et al.*, 2004), makes them an ideal model to study inter-annual variability (*i.e.* temporal stability) of the elemental "tag" for a local nursery ground using otolith microchemistry.

3.1.1 Aims

The aims of this study are outlined as follows:

1. To investigate whether intra-annual (*i.e.* within year) and inter-annual (*i.e.* between years) stability is evident in the elemental signatures of otoliths extracted from juvenile plaice sampled from two recognized plaice nursery grounds in North Wales over a period of seven years between 2004-2010 (excluding 2008).

3.2. Methodology

3.2.1. Juvenile plaice collections

Juvenile plaice were collected over a period of seven years between 2004 and 2010 (excluding 2008 but hereafter referred to as 2004-2010) from two recognized nursery grounds in North Wales: Llanfairfechan (Grid reference SH 67791 75665) and Llanddona (Grid reference SH 56867 81100), (Dunn and Pawson, 2002; Fox *et al.*, 2007; Figure 3. 1.). The nursery ground at Llanfairfechan is a marine coastal area on the North Wales coast, and is situated near Morfa Madryn, a salt marsh nature reserve. The Afon (river) Llanfairfechan runs through Nant y Coed nature reserve above the town of Llanfairfechan and drains into marsh lands to the south and west and onto the shore of Traeth Lafan at Llanfairfechan. The nursery ground at Llandonna is a long sandy beach situated at the eastern end of Red Wharf Bay between Benllech and Penmon on the east coast of Anglesey. Between 2004 and 2007 fish were collected regularly (not every month, but most months of the year, weather-dependent) from the same area (see rectangles in Figure 3.2.1.) in the wave zone and subtidally at each of the two sites. The samples consisted of 0-group and 1-group plaice retained from a previous Ph.D. project (Al-Rashada, 2009, unpublished data). Approximately 10-20 1-group plaice were selected from these retained samples (where 1-group plaice were collected in that month / year) for use in this project. Fish collected during 2009-2010 were all 1-group and were sampled in the months of July (Llanfairfechan) and August (Llanddona) 2009 and October 2010 (both sites).

All fish were collected using a push net (see Wilding *et al.*, 2001 for methods of use), euthanized using a Home Office Schedule 1 method, placed into plastic bags and immediately stored on ice within a portable refrigeration unit (Electrolux F400) for transportation back to the Nuffield Fish Laboratory. The standard length (SL) was measured to the nearest 1.0mm and weight to the nearest 1.0g for each fish, for each year and site (see Table 3.1.) and stored frozen at -30 °C prior to their otolith extraction.

Table 3.1. Details of the standard length (SL) size ranges and their mean ($\bar{x}$) ± 1 standard deviation (sd) for juvenile *Pleuronectes platessa* from two nursery grounds in NW wales caught between 2004 and 2007.

	Llanfairfechan			Llanddona		
Year	**n**	**SL Length (mm)**		**Year**	**n**	**SL Length (mm)**
		Range	**$\bar{x}$ ± sd**			**Range**	**$\bar{x}$ ± sd**
2004	18	18-112	56 ±29	2004	20	12-97	43 ±23
2005	19	14-129	65 ±30	2005	17	20-124	58 ±30
2006	20	17-116	63 ±31	2006	21	23-118	64 ±29
2007	11	37-147	70 ±33	2007	7	42-147	87 ±39
2009	20	52-78	55 ±11	2009	20	53-84	47 ±11
2010	20	44-83	64 ± 8	2010	20	40-86	65 ± 7

3.2.2. Equipment preparation

All equipment used in this study was prepared using the methods outlined in detail in section 2.2.2.

3.2.3. Extraction of otoliths

Otoliths were extracted using the methods outlined in detail in section 2.2.6. with the following modification: juvenile plaice were removed from the freezer in batches of 10 and allowed to thaw for approximately 10 minutes to allow the brain case to defrost prior to otolith removal.

3.2.4. Otolith dilution (Parts I and II)

The plaice otoliths were prepared for measurement using sb-ICP-MS as outlined in detail in sections 2.2.7. and 2.2.7.1. with the following modifications: Due to their small size, some changes in the volumes used to digest the plaice otoliths were made (Dr. Chenery *Pers comm.*, 2010). In the first stage of dilution, 1 ml of digesting acid (16.7% HNO_3 & 6.4% HCl) was initially pipetted and left to allow time for the otolith to effervesce. After the initial effervescing had subsided a second 1 ml of digesting acid solution was pipetted into the partly digested otolith sample ensuring any residue is rinsed back into the bottom of the tube. After a further 20 minutes to allow the otolith to digest and the effervescing to subside, the otolith-acid solution was brought to a final volume of 5 ml by adding 3 ml of Milli Q.

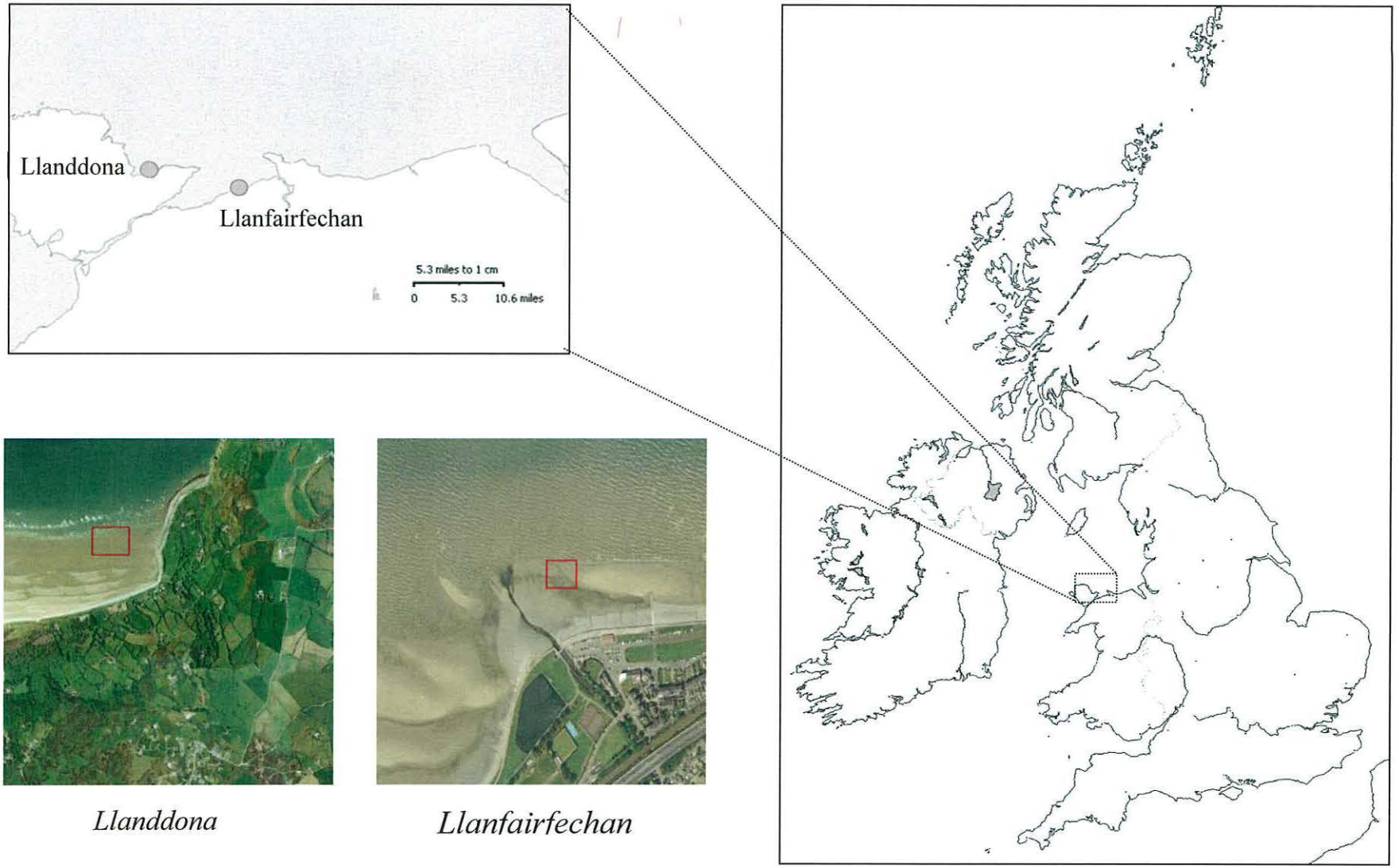


Figure 3.1. Map showing the sampling sites at Llanddona and Llanfairfechan, North Wales where plaice were caught during this study. Rectangles indicate exact sampling location at each of the two sites for the juvenile plaice during 2004-2010.

3.3. sb-ICP-MS data processing

3.3.1. Data screening

The sb-ICP-MS data were initially screened using the methods outlined in detail in section 2.3.1. with the following modifications: 1) due to possible polyatomic interference (Dr. Chenery *Pers comm.*, 2010) two of the 23 elements analysed (Fe and As) were subsequently removed (See Table 3.2.1.1.), 2) In addition the "rare earth" element La was omitted from the final data set due to its low concentration observed within the natural environment and the unlikely occurrence of this element being found within the juvenile plaice otoliths (Dr. Chenery *Pers comm.*, 2010).

3.3.2. Analytical figures of merit including limits of detection

The methods used to assess the limits of detection (LOD) are outlined in detail in section 2.3.2., with the following modifications: After performing the elemental screening, those isotopes which fell below the limits of detection (Li, Al, Cu, Zn, $Cd^{111, 114}$, Sn, $Pb^{206, 207, 208}$, and U) for the site at Llanfairfechan, including Mn and Rb for the site at Llandonna were subsequently omitted from any further analysis (Table 3.2.). In total seven elements (Na, Mg, K, Mn, Rb, Sr and Ba) at the site at Llanfairfechan and five elements (Na, Mg, K, Sr and Ba) at the site at Llandonna were found above the LOD.

Table 3.2.The elements tested (n = 23) for in the initial raw data output for the juvenile plaice otoliths from both Llanddona and Llanfairfechan nursery grounds in descending order of their relative atomic mass. Polyatomic* and elements observed at their limits of detection# (LOD) using a multi-element solution standard and internal standards of the ICP-MS during the sample analysis are indicated.

Elements				
$Li^{\#}$	K	$Cu^{\#}$	Sr	**La**
Na	$^{42,44}Ca$	$Zn^{\#}$	$^{111,114}Cd^{\#}$	$^{206, 207, 208}Pb^{\#}$
Mg	$Mn^{\#}$	As^{*}	$Sn^{\#}$	$U^{\#}$
$Al^{\#}$	Fe^{*}	$Rb^{\#}$	Ba	

Element in **bold** is a rare Earth element and would not be observed above the LOD within the samples (Dr. Chenery *Pers comm.*, 2010).

3.3.3. Elemental outliers & corrections

The methods used to test for outliers in the data set and to correct for them are outlined in detail in section 2.3.3., with the following modifications: After performing the Grubbs and Winsorisation analyses the raw output for each of the remaining elements for the site at Llanfairfechan and the site at Llandonna were assessed for their mean, median and standard deviation and % relative standard deviation (%SRD). As outlined in section 2.3.3., %RSD values between 3-20% are considered optimal to include an element within the data analysis. However, four elements at Llanfairfechan (K, Mn, Rb and Ba) and five elements at Llanddona (Na, Mg, K, Sr and Ba) fell outside the preferred optimal range.

Increases in the %RSD can occur when the measured concentrations of an isotope begin to approach its detection limit (see section 2.3.2.), with the elemental concentrations measured within a sample greater than expected from the quality control solutions and in turn can then exceed that which is desired for analysis (> 20%). However, two of the elements for the site at Llanfairfechan (Mn and Rb) indicated %RSD values greater than 50%, with a further two elements (K and Ba) for the site at Llanddona just below or equal to 50% - K 49% and Ba 50% for the year 2004.

As described in section 2.3.2. if the variation within the sample otoliths are greater than the analytical variation from the quality control solutions useful data can still be obtained even when the %RSD exceeds the optimal desired (>20%), if the between otolith variation is greater than the within. Therefore, to allow for those elements which are used most in biogeochemical analysis in identifying spatial and temporal variability, *i.e.* Mg, Mn, Sr and Ba, and to allow for analyses to be performed on the samples at Llanddona, the %RSD optimal range was extended to include the two elements (K and Ba) at Llandonna for the purpose of this particular study and to increase the suite of elements being assessed between the years (Dr. Chenery *Pers comm.*, 2012). The five elements - Na, Mg, K, Sr and Ba - for the sites at Llanfairfechan and Llanddona were then normalized to calcium using elemental:Ca ratios (Campana, 1999; Thresher, 1999; Elsdon and Gillanders, 2004).

3.4. Statistical analyses

3.4.1. Inter-annual analyses

Tests of normality and homogeneity of variance of the inter-annual data were conducted using the detailed methods outlined in section 2.4.1., additionally methods using both parametric and non-parametric analyses including discriminant function analysis (DFA) are out lined in detail in sections 2.4.2. and 4.4.2. (DFA): Four elements for the site at Llanfairfechan - Na, Mg, Sr and Ba - met the assumptions of normality and equal variance without Log_{10} transformation. However, four elements at Llanddona - Na, K, Sr and Ba – only met the assumptions of equal variance (Levene's $P > 0.05$) after Log_{10} transformation. These elements at the two sites were assessed using standard parametric analyses (*e.g.* ANOVA, GLM, and LDFA). However, two elements failed the assumptions of equal variance, K at Llanfairfechan and Mg at Llanddona, and were therefore assessed using non-parametric tests (*e.g.* Kruskal-Wallis, Mann-Whitney U) and excluded from any further analyses using parametric statistics.

To assess the possible effects of freshwater influx (as salinity ppt) on the elemental concentrations measured from the juvenile plaice otoliths, a Pearson's product-moment correlation analysis was conducted to compare each element between concentrations observed in a given year and the average annual salinity for that year. Salinity data for the analysis was obtained from Natural Resources, Wales from both the nursery grounds at Llanfairfechan (for the years 2006-2010) and Llanddona (for the years 2004-2010).

3.4.2. Intra-annual analyses

In addition to the assessment of inter-annual variability, elemental:Ca ratio data for both sites were assessed for normality and homogeneity of variance following separation into seasonal (spring, summer, autumn and winter) and monthly data sets to allow within year (*i.e.* intra-annual) analyses to be performed. This subdivision of the data sets allowed the inclusion in parametric statistics for the within year study of the elements K (month and season) at Llanfairfechan and the element Mg (month) at Llanddona, with non-parametric analysis used for the remaining elements.

3.4.3. Assessment of right and left otoliths

To determine whether left or right sagittal otoliths differ in their elemental concentrations (see Campana *et al.*, 2000; Secor *et al.*, 2001) and to identify whether the right or left sagittal otolith could have been used for the experiment, 20 randomly chosen left otoliths (10 per site) were run on the ICP-MS in addition to the right otolith measurements. Left otoliths were measured, screened and assessed using the same protocols used for the right otoliths. The elements (converted to their element:Ca ratio) meeting the criteria for their LOD and %RSD for Llanfairfechan (Na, Mg, K, Sr and Ba) and Llanddona (Na, Mg, K, Sr and Ba) were then tested against the data from their opposite right otolith using a paired t-test.

All parametric and non-parametric statistical analyses for the Llanfairfechan and Llanddona data were performed using Minitab v.14.

3.5. Results

3.5.1. Paired otolith analysis

The paired *t*-test results indicated no significant differences in the elemental concentrations between the right and left sagittal otoliths of juvenile plaice at Llanfairfechan (Table 3.3.) or Llanddona (Table 3.4.) for the five elements tested (all $P > 0.05$).

Table 3.3. Paired *t*-tests comparing the elemental:Ca ratio ($x10^{-3}$) in the right and left sagittal otoliths of juvenile plaice for the site at Llanfairfechan. Left and right otoliths are shown as mean ($\bar{x}$) ± 1 standard deviation (sd).

		Left Otolith	Right Otolith		
Element	**n**	$\bar{x} \pm$ sd	$\bar{x} \pm$ sd	**t**	***P***
Na	10	7.479 ±0.953	7.272 ±0.846	0.90	0.390
Mg	10	0.102 ±0.015	0.082 ±0.030	1.57	0.151
K	10	1.479 ±0.256	1.397 ±0.516	0.38	0.712
Sr	10	4.188 ±1.563	4.253 ±0.555	0.14	0.891
Ba	10	0.007 ±0.003	0.006 ±0.001	0.59	0.571

Table 3.4. Paired t- tests comparing the elemental:Ca ratio ($x10^{-3}$) in the right and left sagittal otoliths of juvenile plaice for the site at Llanddona. Left and right otoliths are shown as mean ($\bar{x}$) ± 1 standard deviation (sd).

		Left Otolith	Right Otolith		
Element	**n**	$\bar{x} \pm$ sd	$\bar{x} \pm$ sd	**t**	***P***
Na	9	7.484 ±0.757	7.516 ±0.626	0.16	0.877
Mg	9	0.105 ±0.015	0.104 ±0.041	0.09	0.933
K	9	1.644 ±0.378	1.556 ±0.690	0.37	0.721
Sr	9	4.096 ±1.619	4.741 ±0.578	1.37	0.207
Ba	9	0.008 ±0.004	0.007 ±0.002	0.56	0.591

3.5.a. Llanfairfechan

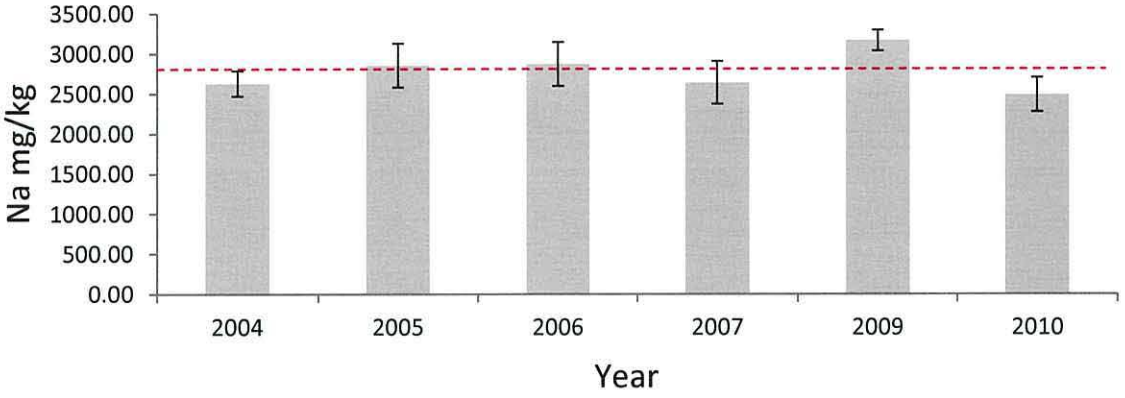
3.5.2. Analysis of Llanfairfechan otoliths

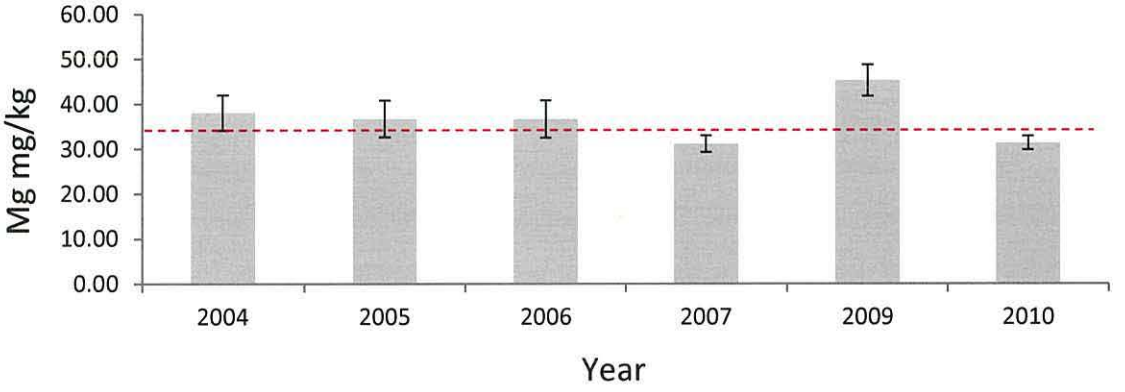
The inter-annual variation in mean concentrations of otolith Na, K, Mg, Sr and Ba for plaice from Llanfairfechan between the years 2004-2010 are presented in Figure 3.2. Inter-annual differences in mean concentration were observed for most elements, most notably for Mg and K (2009 vs. other years), Sr (comparing 2007 and 2010 with the other years) and Ba (comparing 2004 and 2010 with other years) (Figure 3.2.).

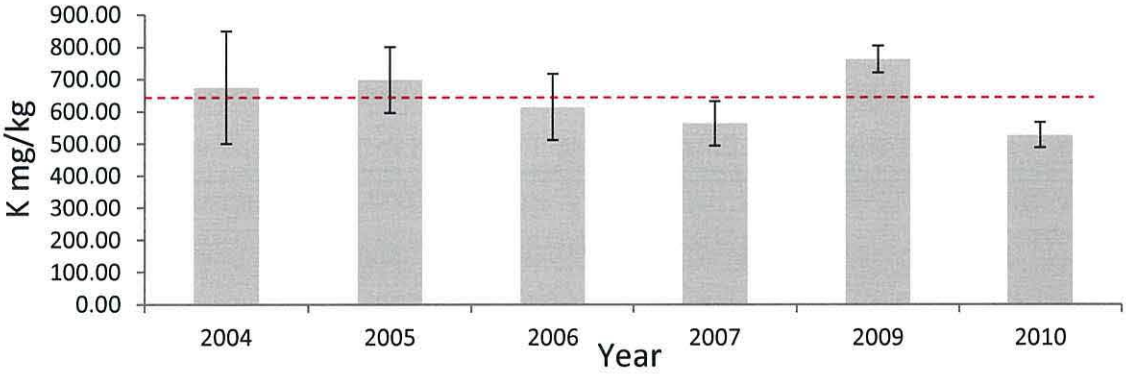
Results of the non-parametric analysis of the K:Ca data showed K concentrations in the otoliths were significantly different between years (Kruskal-Wallis $_{5, 97} = 49$; $P < 0.001$, Table 3.5.). Pairwise comparisons between years using Mann-Whitney tests (Table 3.6.) indicated 10/15 pairwise comparisons were different. Significant differences were observed between 2005 and most other years, between 2004 and 2007 and 2010, and between the year 2006 and 2009 and 2010 (Table 3.6.).

The remaining four elements - Na, Mg, Sr and Ba - were assessed using MANOVA to assess differences observed in their elemental concentrations between 2004-2010. Results indicated significant differences in elemental concentrations for one or more of the four elements and between one or more of the six years ($F_{20, 292} = 12.133$; $P < 0.001$). Individual ANOVAs (run in conjunction with MANOVA) indicated significant inter-annual differences in elemental concentrations for all 4 elements; Na ($F_{5, 96} = 23.04$; $P < 0.001$), Mg ($F_{5, 96} = 36.63$; $P < 0.001$), Sr ($F_{5, 96} = 32.12$; $P < 0.001$) and Ba ($F_{5, 96} = 4.64$; $P < 0.001$).

Multiple *post-hoc* pairwise comparisons (Bonferroni test) indicated significant ($P < 0.05$) or highly significant ($P < 0.001$) differences between years for Na, Mg, Sr and Ba (see Table 3.7.). Significant differences were observed in 7/15 pairwise comparisons for Na between 2009-2010 and the earlier years 2004-2007. Mg differed significantly in 11/15 pairwise comparisons between 2007-2010 and the earlier years 2004-2006. Sr indicated significant differences in 12/15 pairwise comparisons and especially between 2004 and all years (excluding 2009), and comparing 2005-2006 with subsequent years 2007-2010 (Table 3.7.). However, Ba indicated the least significant inter-annual variation in otolith concentrations with 2/15 pairwise comparisons significant between 2004 and 2009, and 2009 and 2010 (Table 3.7.).







(Figure 3.2. and caption are continued overleaf)

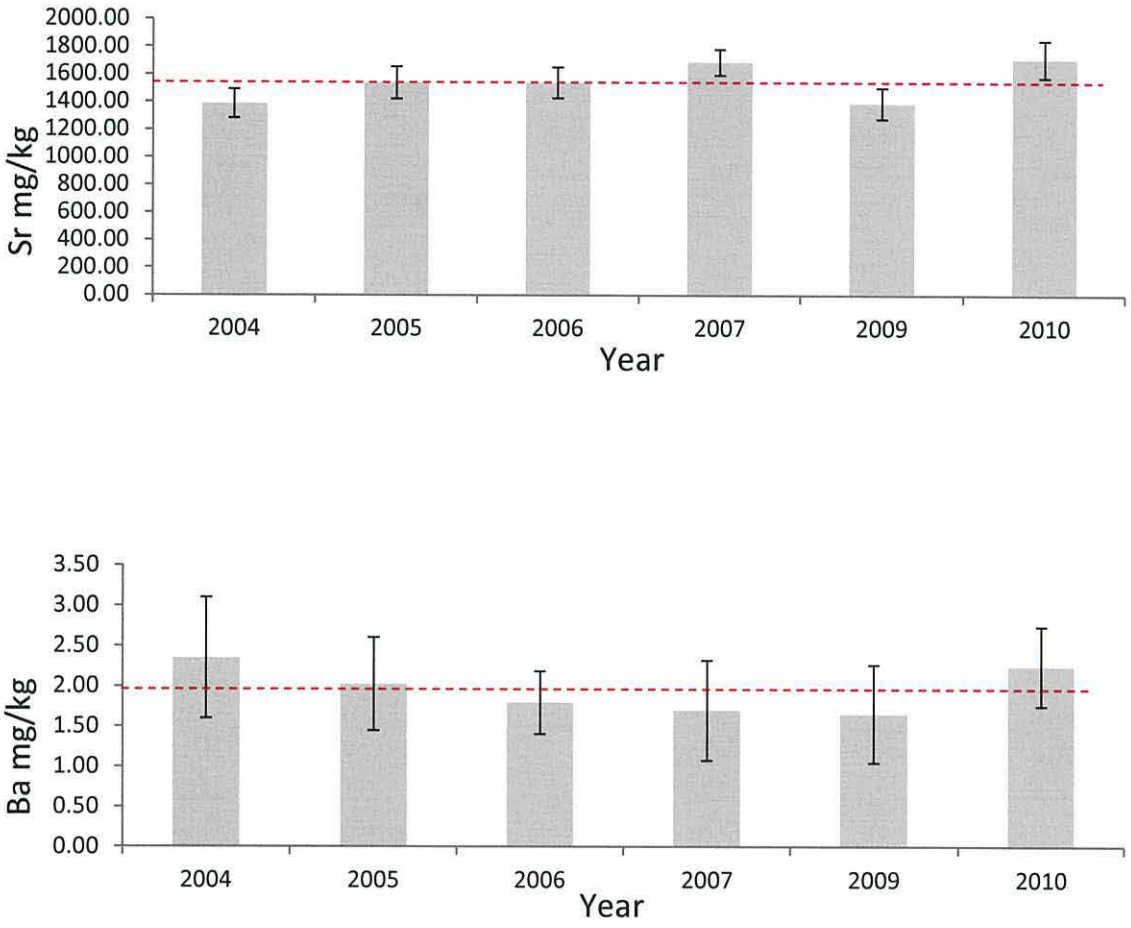


Figure 3.2. Mean (± 1 standard deviation) elemental concentrations (mg/kg) for Na, Mg, K, Sr and Ba in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan for the years 2004-2010 (excluding 2008). The dashed line indicates the long term average value calculated from the combined data set.

Table 3.5. Inter-annual differences in K:Ca ratio in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan for the years 2004-2010 (excluding 2008) assessed using the non-parametric Kruskal-Wallis test.

K	n	DF	Median	Average Rank	Z value	P
Year						
2004	13		0.00162	58.5	1.31	< 0.001
2005	17		0.00182	62.9	2.24	
2006	17		0.00157	42.8	-1.01	
2007	11		0.00145	29.3	-2.47	
2009	19		0.00198	76.1	4.67	
2010	20		0.00139	21.5	-4.91	
Overall	97	5		49		

Table 3.6. Pairwise comparisons assessed using Mann-Whitney U test of K:Ca ratio concentrations in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan for the years 2004-2010 (excluding 2008). Significant differences are highlighted in bold and are at the α 0.05 level.

						95% C.I.		
Year	**Median**	**Year**	**n**	**Median**	**W**	**Lower**	**Upper**	**P**
2004	0.00162		13					
		2005	17	0.00182	198.0	-0.00033	0.00030	0.900
		2006	17	0.00157	233.0	-0.00004	0.00052	0.195
		2007	11	0.00145	197.0	0.00000	0.00069	**0.049**
		2009	19	0.00198	190.0	-0.00044	0.00012	0.357
		2010	20	0.00139	307.0	0.00012	0.00069	**0.002**
2005	0.00182	2006	17	0.00157	365.0	0.00004	0.00044	**0.021**
		2007	11	0.00145	319.0	0.00016	0.00056	**0.001**
		2009	19	0.00198	250.0	-0.00034	0.00001	**0.043**
		2010	20	0.00139	480.0	0.00025	0.00058	**< 0.001**
2006	0.00157	2007	11	0.00145	275.0	-0.00005	0.00029	0.188
		2009	19	0.00198	184.0	-0.00051	0.00032	**< 0.001**
		2010	20	0.00139	418.0	0.00006	0.00026	**0.004**
2007	0.00145	2009	19	0.00198	66.0	-0.00064	-0.00041	**< 0.001**
		2010	20	0.00139	199.0	-0.00006	0.00016	0.353
2009	0.00198	2010	20	0.00139	570.0	0.00051	0.00064	**< 0.001**

Table 3.7. Summary of the *post-hoc* pairwise comparisons (using a Bonferroni test) comparing the element:Ca ratios of Na, Mg, Sr and Ba in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan between the years 2004-2010 (excluding 2008). Significant differences at the α 0.05 level are indicated in **bold**.

Na	2004	2005	2006	2007	2009	2010
2010	0.706	**0.0001**	**0.0001**	1.000	**< 0.001**	
2009	**< 0.001**	**0.0001**	**0.0001**	**< 0.001**		
2007	1.000	0.059	0.066			
2006	0.370	1.000				
2005	0.334					
2004						

Mg	2004	2005	2006	2007	2009	2010
2010	**<0.001**	**0.0014**	**0.0036**	1.000	**<0.001**	
2009	**<0.001**	**<0.001**	**<0.001**	**<0.001**		
2007	**<0.001**	**0.009**	**0.0021**			
2006	0.878	1.000				
2005	1.000					
2004						

Sr	2004	2005	2006	2007	2009	2010
2010	**< 0.001**	**< 0.001**	**< 0.001**	1.000	**< 0.001**	
2009	1.000	**0.006**	**0.019**	**< 0.001**		
2007	**< 0.001**	**0.009**	**0.038**			
2006	**0.047**	1.000				
2005	**0.020**					
2004						

Ba	2004	2005	2006	2007	2009	2010
2010	1.000	1.000	0.137	0.112	**0.012**	
2009	**0.009**	0.833	1.000	1.000		
2007	0.065	1.000	1.000			
2006	0.081	1.000				
2005	1.000					
2004						

Mean concentrations of otolith Na, K, Mg, Sr and Ba are presented in Figure 3.3. for each month where sampling took place in each of the six years (2004-2010). Similar to the variation in elemental concentrations observed in Figure 3.2., mean concentrations of each element between each sampling month (and year) indicated possible intra-annual elemental differences. The possible effects of intra-annual variability observed in elemental concentrations between months within years (Figure 3.3.) and subsequently their possible contribution to the effects / variability observed in elemental concentrations for the inter-annual data (Figure 3.2.), were assessed using ANOVA.

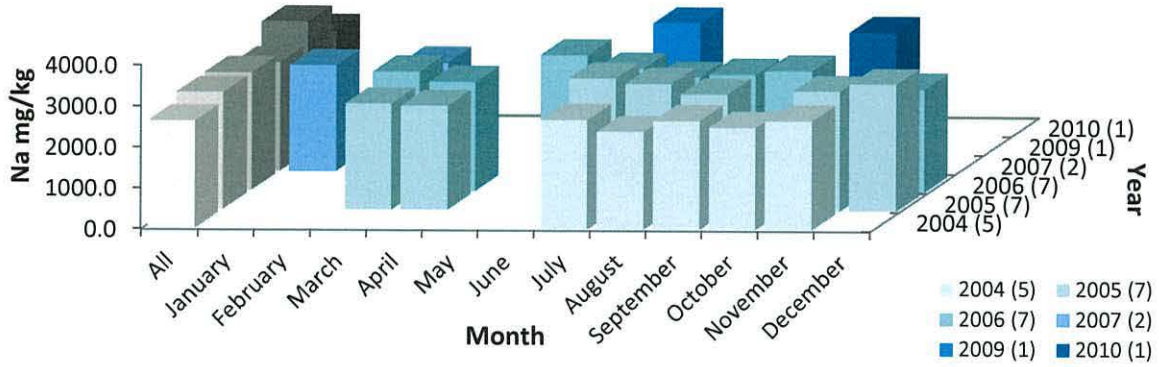
Due to the lack of sampling consistency between years (*i.e.* sampling months varied between all years see Figure 3.3.) and the possible effect of intra-annual variability (*i.e.* sample month and year) when sampling at different periods within years, each month was assessed separately using ANOVA to address inter-annual changes in elemental concentrations between years containing the same sample month.

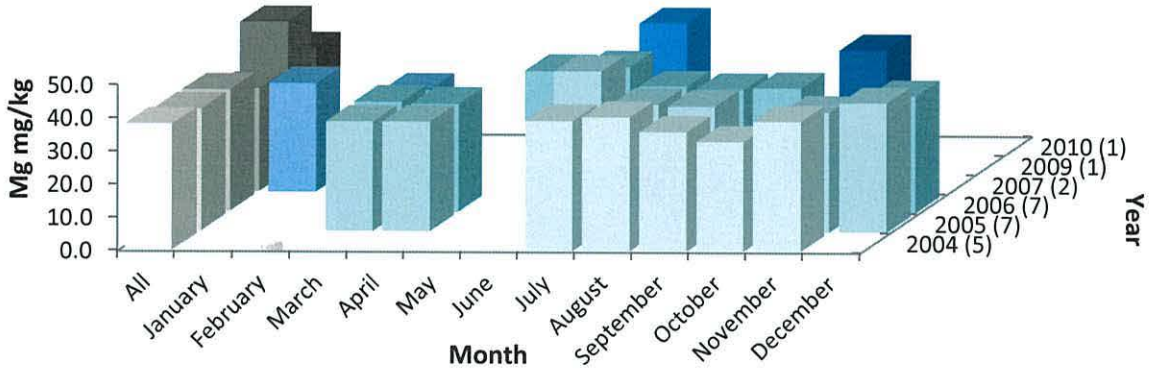
Significant differences were observed in the elemental compositions between years for the sample months of March (Sr; 2005-2007), July (Na and Ba; 2004, 2006, and 2009) and October (Na, Mg, Sr and Ba; 2004, 2006 and 2010) see Table 3.8. However, no significant variation in elemental composition was observed for any of the five elements Na, Mg, K, Sr and Ba for the month of September between the years 2004-2006 ($P > 0.05$ Table 3.8.).

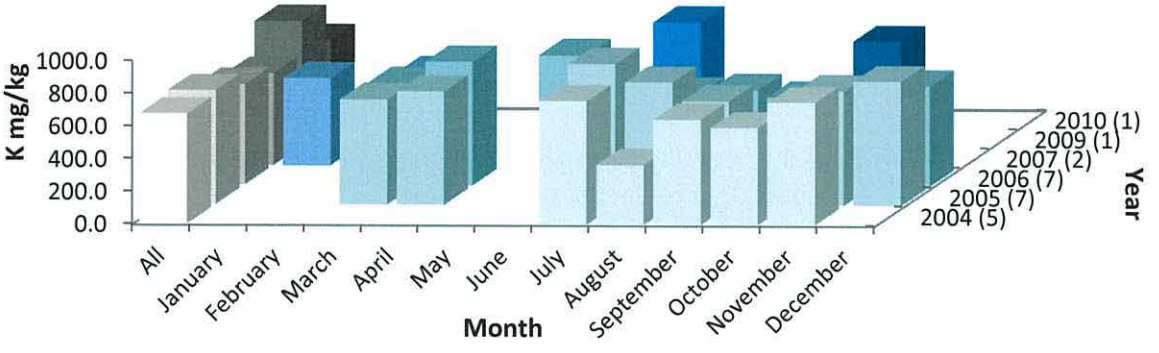
Table 3.8. Results of ANOVA analyses to examine otolith element:Ca ratios in the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan caught in the same month but in different sampling years. Significant differences at the α 0.05 level are indicated in **bold**.

Month (Year)	n	Na		Mg		K		Sr		Ba	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
March (2005-2007)	15	1.16	0.346	1.92	0.190	1.10	0.364	4.40	**0.037**	1.34	0.229
July (2004,2006,2009)	23	7.57	**0.002**	1.37	0.282	*	*	0.24	0.866	5.90	**0.005**
September (2004-2006)	9	0.89	0.459	0.22	0.811	0.20	0.823	2.97	0.127	0.48	0.639
October (2004,2006,2010)	25	6.82	**0.005**	10.06	**0.001**	1.20	0.321	6.45	**0.006**	4.51	**0.023**
November (2004-2005)	11	3.09	0.113	2.47	0.150	0.73	0.414	1.67	0.228	0.29	0.603

* Element failed assumptions of normality and equal variance after Log_{10} transformation







(Figure 3.3. and caption are continued overleaf)

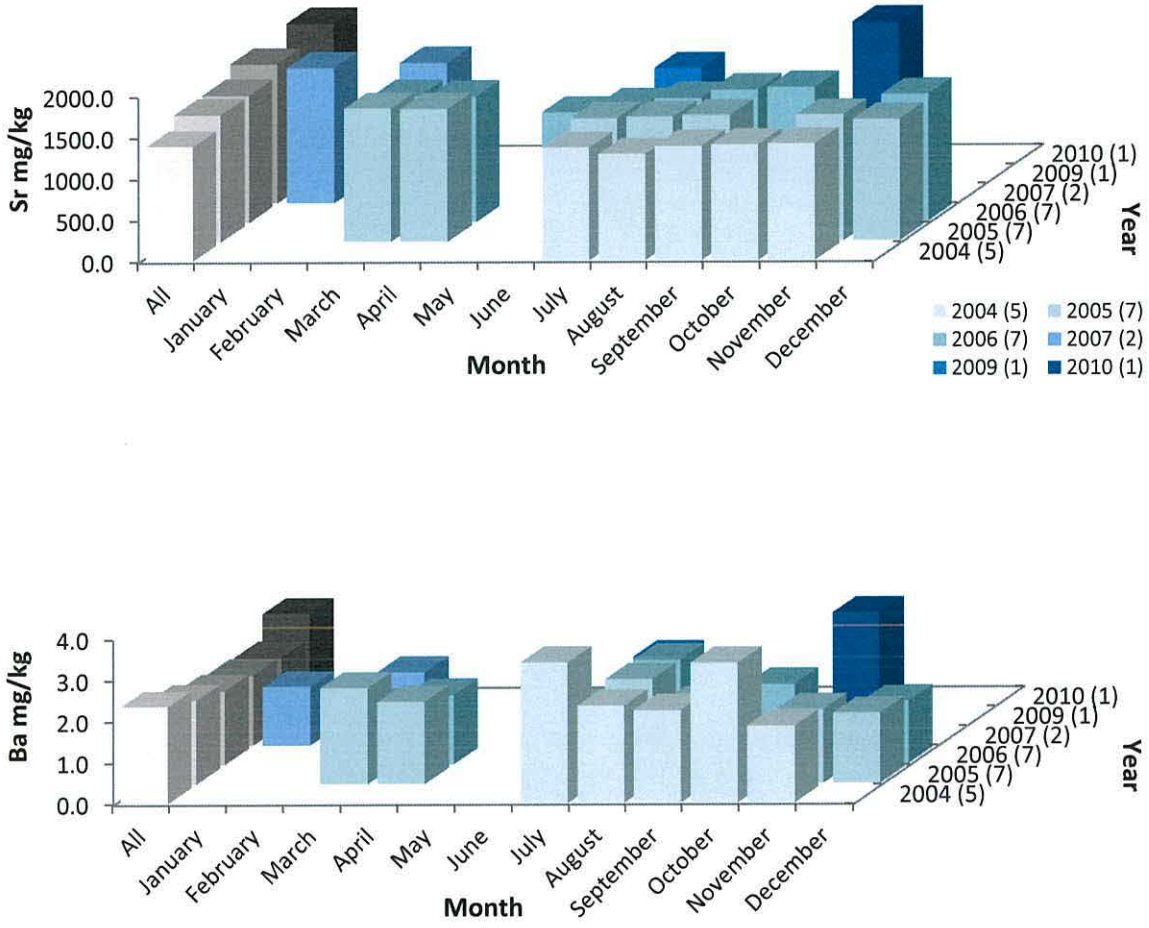


Figure 3.3. Mean element concentrations (mg/kg) for Na, Mg, K, Sr and Ba in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan for the years 2004-2010 (excluding 2008; see table 3.1. for juvenile plaice sample numbers for each year). Total mean concentrations for the pooled months for each year are described by **All**. Number of months per year are in parenthesis.

Table 3.9. Monthly periods pooled into their respective seasonal categories for the analysis at Llanfairfechan.

Month	Season
March - May	Spring
June - August	Summer
September - November	Autumn
December - February	Winter

Due to the lack of sampling consistency observed between years (*i.e.* sampling months varied between years) and to include all months sampled but not necessarily analysed between years (see Figure 3.3.2 and Table 3.8.), months were pooled together into their respective seasons (Table 3.9.) and measured between all years.

ANOVAs run for all years showed significant inter-annual differences in the otolith elemental concentrations of Na ($F_{5, 96} = 4.48$; $P < 0.001$) and Mg ($F_{5, 96} = 10.15$; $P < 0.001$) between seasons. However, the elemental concentrations of K ($F_{5, 96} = 0.52$; $P = 0.67$), Sr ($F_{5, 96} = 1.37$; $P = 0.26$) and Ba ($F_{5, 96} = 2.71$; $P = 0.05$) showed no variation between season. Na showed a similar trend with season to the monthly analysis in that summer (July) were indicating significant changes in chemical composition (Table 3.10.). Similarly, Mg showed an effect of month (October) and season (autumn) on the chemical composition for both tests. Season reduced the significance of the month October for Na but increased the effect of spring for Mg (see Table 3.10.).

Using a Pearson's product-moment correlation to assess possible effects of salinity and elemental concentration in a given year, the results indicated no significant correlation between possible freshwater influence and changes in the elemental concentrations for the five elements Na, Mg, K, Sr and Ba for the years 2006-2010 (all P > 0.05 see Table 3.11.).

Table 3.11. Effects of salinity (river influx) and element:Ca ratios ($x10^{-3}$) of Na, Mg, K, Sr and Ba (using Pearson's correlation) for each of the years 2006-2010 (excluding 2008) at the nursery ground at Llanfairfechan. Elements and salinity are shown as mean ($\bar{x}$) values for each year.

Element	2006	2007	2009	2010	R	*P*
Na	7.468	6.882	8.303	6.659	-0.194	0.806
Mg	0.096	0.081	0.119	0.084	0.002	0.998
K	1.598	1.469	1.999	1.411	-0.136	0.864
Sr	4.009	4.409	3.648	4.586	0.256	0.744
Ba	0.0047	0.0045	0.0043	0.0060	0.864	0.136
$\bar{x}$ **Salinity**	32.0	31.2	32.2	33.7	-	-

No salinity data was available for 2004-2005

Table 3.10. Summary of the *post-hoc* pairwise comparisons (using Bonferroni tests) comparing the elemental concentrations of the element:Ca ratios for Na, Mg, K, Sr and Ba between seasons (all years combined) at the nursery ground at Llanfairfechan. Significant differences at the α 0.05 level are indicated in **bold**.

Na	Spring	Summer	Autumn	Winter
Winter	1.000	0.659	1.000	
Autumn	0.109	0.519		
Summer	**0.003**			
Spring				

Mg	Spring	Summer	Autumn	Winter
Winter	0.740	0.359	1.000	
Autumn	**0.013**	**0.020**		
Summer	**< 0.001**			
Spring				

K	Spring	Summer	Autumn	Winter
Winter	1.000	1.000	1.000	
Autumn	1.000	1.000		
Summer	1.000			
Spring				

Sr	Spring	Summer	Autumn	Winter
Winter	1.000	1.000	1.000	
Autumn	1.000	0.839		
Summer	0.967			
Spring				

Ba	Spring	Summer	Autumn	Winter
Winter	1.000	0.393	1.000	
Autumn	1.000	0.059		
Summer	0.640			
Spring				

Monthly analysis within year (intra-annual) for the elements Na, Mg, K, Sr and Ba were assessed using MANOVA to measure any differences within the chemical concentrations between months and seasons sampled within the years 2004-2007. Results indicated significant differences in elemental concentrations of one of the five elements between months and seasons for the year 2006 ($P < 0.05$, see Table 3.12.). However, the years 2004-2005 and 2007 indicated no significant differences in the elemental concentrations between months or seasons ($P > 0.05$, see Table 3.12.).

Table 3.12. Results of MANOVA analysis to examine otolith element:Ca ratios in the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan caught in the same year but in different sampling months and seasons. Significant differences at the α 0.05 level are indicated in **bold**.

Year	Analysis	df	F	*P* value
2004				
	Month	20	1.269	0.329
	Season	5	1.958	0.203
2005				
	Month	30	1.236	0.293
	Season	10	1.410	0.245
2006				
	Month	35	2.152	**0.029**
	Season	10	4.121	**0.003**
2007				
	Month	5	2.328	0.188
	Season	5	2.328	0.188

df. Degrees of freedom.

Individual ANOVAs (run in conjunction with MANOVA) indicated significant intra-annual differences in otolith elemental concentrations for 3 elements when looking at months within year; Na (2005; $F_{6, 16} = 3.62$; $P = 0.036$), Mg (2005 and 2006; $F_{6, 16} = 6.95$; $P = 0.004$; $F_{7, 16} = 3.85$; $P = 0.032$ respectively) and Ba (2004; $F_{4, 12} = 5.36$; $P = 0.021$), (see Table 3.13.). Similarly, ANOVAs run between seasons within a year indicated significant differences in the elemental concentrations of 4 elements; Na (2005; $F_{2, 16} = 7.97$; $P = 0.005$), Mg (2006; $F_{2, 16} = 11.79$; $P = 0.001$), Sr (2006; $F_{2, 16} = 4.60$; $P = 0.029$) and Ba (2004; $F_{1, 12} = 5.22$; $P = 0.043$), (see Table 3.13.).

Using multiple pairwise comparisons (Bonferroni test) significant ($P < 0.05$) differences between months within years were observed for the element Mg (Table

3.14.). Significant differences were observed in 3/21 pairwise comparisons for Mg between 2005 and the months March, April and July (see Table 3.14.). Multiple pairwise comparisons (Bonferroni test) between seasons and year indicated significant ($P < 0.05$) differences in otolith elemental concentrations between seasons within years were observed for of Mg, Sr and Ba (Table 3.15.). Significant differences were observed in 2/3 pairwise comparisons for Na between spring and the seasons summer and autumn for the year 2006 (Table 3.15.). Sr differed significantly for autumn when looking at summer in 2006. Similarly, Ba differed significantly between autumn and summer in the year 2004 See Table 3.15.).

Table 3.13. Results of ANOVA analysis to examine otolith element:Ca ratios in the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) from Llanfairfechan caught in the same year but in different sampling months and seasons. Significant differences at the α 0.05 level are indicated in **bold**.

Year	Analysis	df	F	*P* value
Na				
2004	*Month*	12	0.41	0.798
	Season	12	0.13	0.722
2005	*Month*	16	3.62	**0.036**
	Season	16	7.97	**0.005**
2006	*Month*	16	1.83	0.197
	Season	16	1.42	0.274
2007	*Month*	10	0.23	0.644
	Season	10	0.23	0.644
Mg				
2004	*Month*	12	1.08	0.429
	Season	12	0.66	0.435
2005	*Month*	16	6.95	**0.004**
	Season	16	3.56	0.056
2006	*Month*	16	3.85	**0.032**
	Season	16	11.97	**0.001**
2007	*Month*	10	1.50	0.252
	Season	10	1.50	0.252

df. Degrees of freedom.

Table 3.13. Continued

Year	Analysis	df	F	P value
K				
2004	*Month*	12	0.97	0.475
	Season	12	0.27	0.615
2005	*Month*	16	0.87	0.548
	Season	16	0.42	0.666
2006	*Month*	16	3.27	0.051
	Season	16	2.04	0.168
2007	*Month*	10	0.58	0.465
	Season	10	0.58	0.465
Sr				
2004	*Month*	12	0.57	0.695
	Season	12	0.57	0.466
2005	*Month*	16	0.67	0.677
	Season	16	2.54	0.114
2006	*Month*	16	1.40	0.311
	Season	16	4.60	0.029
2007	*Month*	10	0.92	0.361
	Season	10	0.92	0.361
Ba				
2004	*Month*	12	5.36	**0.021**
	Season	12	5.22	**0.043**
2005	*Month*	16	0.51	0.789
	Season	16	0.42	0.663
2006	*Month*	16	3.02	0.063
	Season	16	2.21	0.147
2007	*Month*	10	0.46	0.516
	Season	10	0.46	0.516

df. Degrees of freedom.

Table 3.14. Summary of the Intra-annual *post-hoc* pairwise comparisons (using Bonferroni tests) comparing the elemental concentrations of the element:Ca ratios for Na, Mg, K, Sr and Ba between months within years at the nursery ground at Llanfairfechan. Significant differences at the α 0.05 level are indicated in **bold**.

Na					**Month**					
Year		**Mar**	**Apr**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	July					1.0000	1.0000	1.0000	1.0000	
	Aug						1.0000	1.0000	1.0000	
	Sept							1.0000	1.0000	
	Oct								1.0000	
2005	Mar		1.0000		0.3308	0.3420	1.0000		0.9031	1.0000
	Apr				0.2137	0.2172	1.0000		0.5193	0.6769
	July					1.0000	1.0000		1.0000	1.0000
	Aug						1.0000		1.0000	1.0000
	Sept								1.0000	1.0000
	Nov									1.0000
2006	Mar		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000
	Apr			0.3748	1.0000	1.0000	1.0000	1.0000		1.0000
	June				1.0000	1.0000	1.0000	1.0000		0.4618
	July					1.0000	1.0000	1.0000		1.0000
	Aug						1.0000	1.0000		1.0000
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.6440								

Mg					**Month**					
Year		**Mar**	**Apr**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	July					1.0000	1.0000	1.0000	1.0000	
	Aug						1.0000	0.8529	1.0000	
	Sept							1.0000	1.0000	
	Oct								1.0000	
2005	Mar		1.0000		**0.0038**	0.7612	0.6166		1.0000	1.0000
	Apr				**0.0051**	0.8404	0.7330		1.0000	1.0000
	July					0.0974	0.0563		**0.0127**	0.2550
	Aug						1.0000		1.0000	1.0000
	Sept								1.0000	1.0000
	Nov									1.0000
2006	Mar		1.0000	0.1917	0.1295	1.0000	1.0000	1.0000		1.0000
	Apr			0.2492	0.1713	1.0000	1.0000	1.0000		1.0000
	June				1.0000	1.0000	1.0000	1.0000		1.0000
	July					1.0000	1.0000	1.0000		1.0000
	Aug						1.0000	1.0000		1.0000
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.2523								

Table 3.14. Continued

K		Month								
Year		**Mar**	**Apr**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	July					1.0000	1.0000	1.0000	1.0000	
	Aug						1.0000	1.0000	1.0000	
	Sept							1.0000	1.0000	
	Oct								1.0000	
2005	Mar		1.0000		1.0000	1.0000	1.0000		1.0000	1.0000
	Apr				1.0000	1.0000	1.0000		1.0000	1.0000
	July					1.0000	1.0000		1.0000	1.0000
	Aug						1.0000		1.0000	1.0000
	Sept								1.0000	1.0000
	Nov									1.0000
2006	Mar		1.0000	0.9204	1.0000	1.0000	1.0000	1.0000		1.0000
	Apr			1.0000	1.0000	0.8583	0.9004	0.1599		1.0000
	June				1.0000	0.5314	0.5702	0.1542		1.0000
	July					1.0000	1.0000	1.0000		1.0000
	Aug						1.0000	1.0000		1.0000
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.4654								

Sr		Month								
Year		**Mar**	**Apr**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	July					1.0000	1.0000	1.0000	1.0000	
	Aug						1.0000	1.0000	1.0000	
	Sept							1.0000	1.0000	
	Oct								1.0000	
2005	Mar		1.0000		1.0000	1.0000	1.0000		1.0000	1.0000
	Apr				1.0000	1.0000	1.0000		1.0000	1.0000
	July					1.0000	1.0000		1.0000	1.0000
	Aug						1.0000		1.0000	1.0000
	Sept								1.0000	1.0000
	Nov									1.0000
2006	Mar		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000
	Apr			1.0000	1.0000	1.0000	1.0000	1.0000		1.0000
	June				1.0000	1.0000	1.0000	1.0000		1.0000
	July					1.0000	1.0000	1.0000		1.0000
	Aug						1.0000	1.0000		1.0000
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.3613								

Table 3.14. Continued

Ba		Month								
Year		**Mar**	**Apr**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	July					1.0000	0.3738	1.0000	0.5192	
	Aug						0.2161	1.0000	0.1724	
	Sept							0.1959	1.0000	
	Oct								0.1486	
2005	Mar		1.0000		1.0000	1.0000	1.0000		1.0000	1.0000
	Apr				1.0000	1.0000	1.0000		1.0000	1.0000
	July					1.0000	1.0000		1.0000	1.0000
	Aug						1.0000		1.0000	1.0000
	Sept								1.0000	1.0000
	Nov									1.0000
2006	Mar		1.0000	1.0000	1.0000	0.0666	1.0000	1.0000		1.0000
	Apr			1.0000	1.0000	0.4247	1.0000	1.0000		1.0000
	June				1.0000	1.0000	1.0000	1.0000		1.0000
	July					0.3682	1.0000	1.0000		1.0000
	Aug						0.4584	0.8173		0.5198
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.5161								

Table 3.15. Summary of the Intra-annual *post-hoc* pairwise comparisons (using Bonferroni tests) comparing the elemental concentrations of the element:Ca ratios for Na, Mg, K, Sr and Ba between seasons within years at the nursery ground at Llanfairfechan. Significant differences at the α 0.05 level are indicated in **bold**.

Na 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	Autumn
Winter					**Winter**				
Spring					**Spring**	0.0882			
Summer					**Summer**	1.0000	0.0058		
Autumn			0.7224		**Autumn**				
2006	Winter	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring					**Spring**	0.6440			
Summer		0.3572			Summer				
Autumn		1.0000	0.6997		Autumn				

Table 3.15. Continued

Mg 2004	Winter	Spring	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	Autumn
Winter					**Winter**				
Spring					**Spring**	0.5552			
Summer					**Summer**	1.0000	0.0613		
Autumn			0.4354		Autumn				
2006	Winter	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring					**Spring**	0.2523			
Summer		**0.0008**			Summer				
Autumn		**0.0355**	0.0622		Autumn				

K 2004	Winter	Spring	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	Autumn
Winter					**Winter**				
Spring					**Spring**	1.0000			
Summer					**Summer**	1.0000	1.0000		
Autumn			0.6153		Autumn				
2006	**Winter**	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring					**Spring**	0.4654			
Summer		1.0000			Summer				
Autumn		0.2166	0.7513		Autumn				

Sr 2004	Winter	Spring	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	Autumn
Winter					**Winter**				
Spring					**Spring**	0.4060			
Summer					**Summer**	1.0000	0.1805		
Autumn			0.4663		Autumn				
2006	Winter	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring					**Spring**	0.3613			
Summer		1.0000			Summer				
Autumn		0.2226	**0.0368**		Autumn				

Table 3.15. Continued

Ba 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	Autumn
Winter					**Winter**				
Spring					**Spring**	1.0000			
Summer					**Summer**	1.0000	1.0000		
Autumn			**0.0432**		Autumn				
2006	Winter	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring					**Spring**	0.5161			
Summer		0.1655			Summer				
Autumn		0.6876	0.8438		Autumn				

Temporal stability of elemental concentrations of Na, Mg, Sr and Ba in juvenile plaice otoliths from Llanfairfechan between years (2004-2010) were investigated using linear discriminant function analysis (LDFA). If temporal stability was apparent then classification results looking at year would be poor. Conversely, if classification results were observed to be high, temporal stability between years would therefore not be present.

Percentage correct classification between years was relatively high (63.9%) with 2004 and the years 2007-2010 most notably showing high classification back to year (69.2 - 94.7%). In contrast, 2005 and 2006 showed relatively low classification results of 23.5, 41.2% respectively. Cross-validation (leave on out method) yielded only a slightly reduced classification result for the multi-elemental chemistry of the otoliths (60.3% Table 3.16.).

Multi-elemental analysis of the four elements Na, Mg, Sr and Ba using principal component analysis (PCA) indicated relatively good separation for all years (2004-2010), most notably between 2009-2010 and earlier years (Figure 3.4.). However, years 2004-2007 showed considerable overlap using all four elements (Figure 3.4.), with the elements Na, Mg and Sr explaining 60% of the total variance (PC1) and Ba 24% for PC2.

Table 3.16. Linear discriminant function analysis (LDFA) original classification (top of table) and cross validation classification, leave one out method (bottom table) of juvenile plaice (*Pleuronectes platessa*) collected from Llanfairfechan during 2004-2010 (excluding 2008) using element:Ca ratios of Na, Mg, Sr and Ba. Numbers in **bold** indicate percentage of correctly classified fish to their sample year. Total n = number of individuals analysed with their total accumulated percentage of correctly classified fish in parenthesis.

	Predicted nursery ground Llanfairfechan						
	2004	**2005**	**2006**	**2007**	**2009**	**2010**	**Total *n***
Original Count							
2004	**9 (69.2%)**	2	1		1		13
2005	2	**4 (23.5%)**	7	1	1	2	17
2006	1	4	**9 (41.2%)**	1	2		17
2007		1		**8 (72.7%)**		2	11
2009		1			**18 (94.7%)**		19
2010	1			5		**14 (70%)**	20
							63.9%
Cross Validation Count							
2004	**8 (61.5%)**	2	2		1		13
2005	2	**4 (23.5%)**	7	1	1	2	17
2006	1	5	**7 (23.5%)**	2	2		17
2007		1	1	**6 (54.5%)**		3	11
2009	1	2			**16 (84.2%)**		19
2010	1			5		**14 (70%)**	20
							60.3%

*Light and dark panels indicate adjacent years to which fish were incorrectly classified from their original sample year when using LDFA.

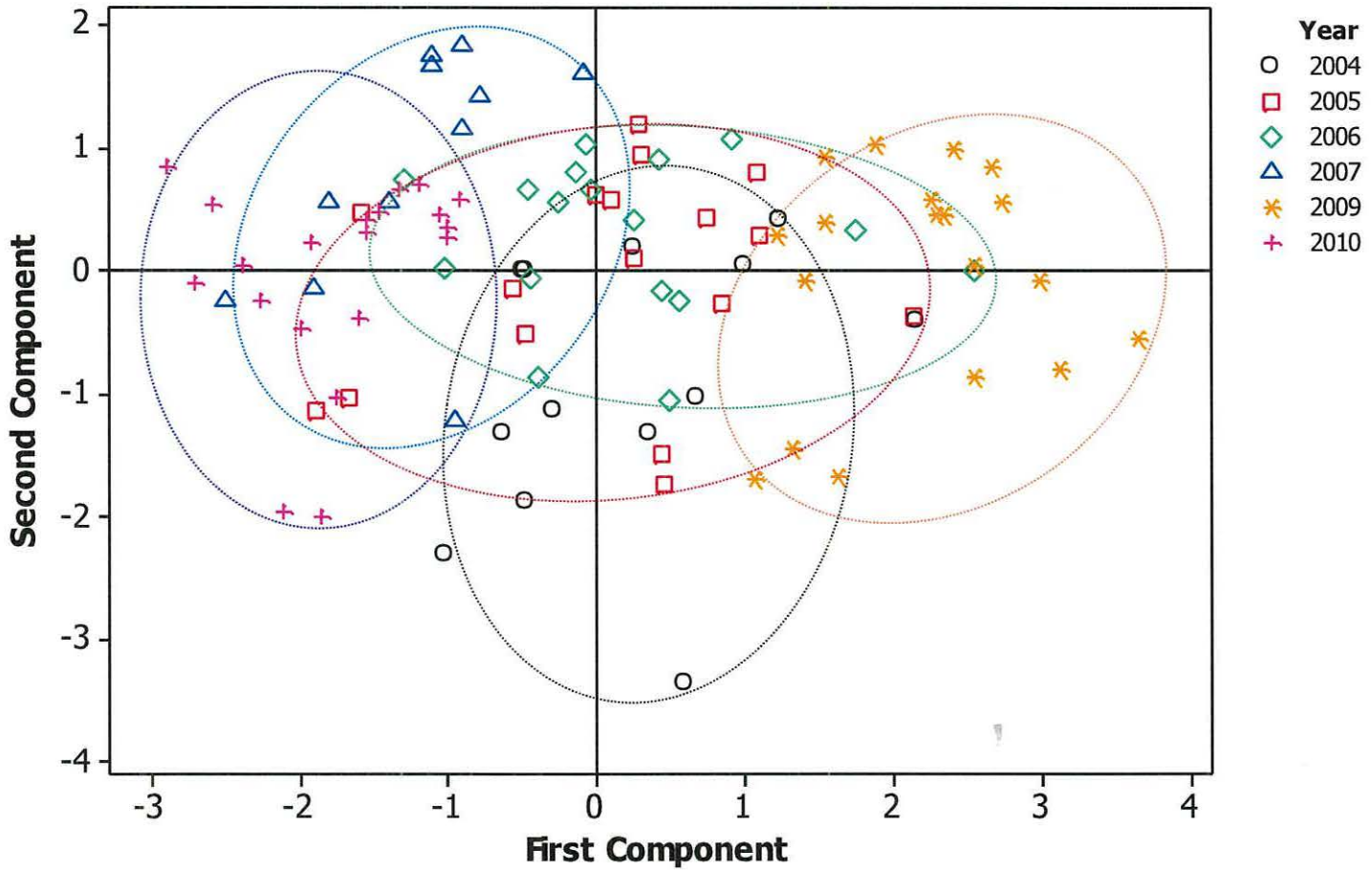


Figure 3.4. PCA displaying year class from the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) collected from Llanfairfechan during 2004-2010 (excluding 2008) using element:Ca ratios of Na, Mg, Sr and Ba. Clustering used to allow year class overlap to be observed.

3.5.b. Llanddona

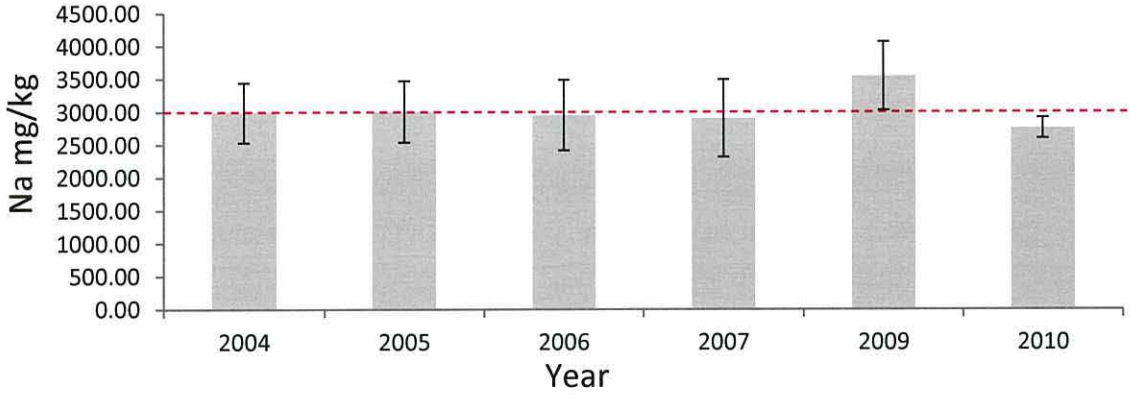
3.5.3. Analysis of Llanddona Otoliths

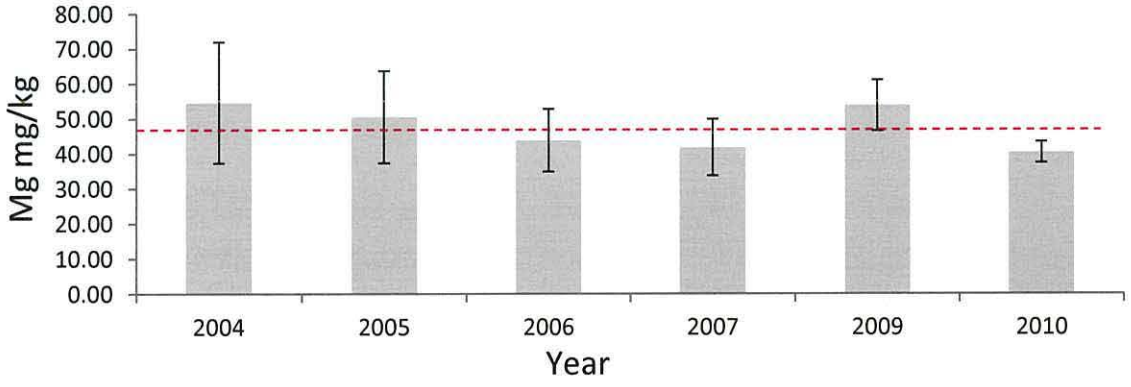
Inter-annual variation in mean elemental concentrations of otolith Na, K, Mg, Sr and Ba for plaice from Llanddona between the years 2004-2010 are presented in Figure 3.5. Inter-annual differences could be observed in the mean concentrations for most elements; most notably when looking at Na and K (2009) compared with other years, Sr (2007 vs. other years) and Ba (comparing 2004-2006 with 2007-2010) (Figure 3.5.).

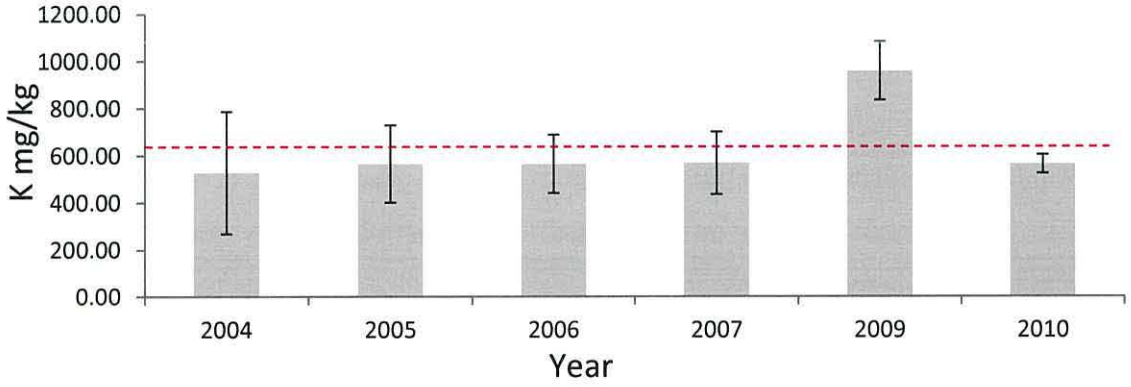
Results of the non-parametric analyses indicated that Log_{10} Mg concentrations in the otoliths were significantly different between years (Kruskal-Wallis $_{5, 92} = 46.5$; $P < 0.001$, Table 3.17.). Pairwise comparisons between years using a Mann-Whitney test (Table 3.18.) indicated 9/15 pairwise comparisons were significant ($P < 0.05$) for Mg between 2006-2007 and 2004, between 2005 and 2009, between 2010 and 2004, and between the year 2010 and 2005, 2009.

The remaining Log_{10} elements (Na, K, Sr and Ba) were assessed using MANOVA to determine any differences observed in their elemental concentrations between 2004-2010. The result indicated a highly significant difference in the elemental concentrations of Na, K, Sr and Ba between one or more of the six years ($F_{20, 259} = 7.479$; $P < 0.001$). Individual ANOVAs (run in conjunction with MANOVA) indicated highly significant differences between years 2004-2010 for all four elements Na ($F_{5, 91} = 8.06$; $P < 0.001$), K ($F_{5, 87} = 22.19$; $P < 0.001$), Sr ($F_{5, 91} = 3.81$; $P = 0.004$) and Ba ($F_{5, 89} = 3.99$; $P = 0.003$).

Multiple pairwise comparisons (Bonferroni test) indicated significant ($P < 0.05$) or highly significant ($P < 0.001$) differences between years for Na, K, Sr and Ba (Table 3.19.). Na concentrations differed significantly between years for 7/15 pairwise comparisons; between 2007/2010 and years 2004/2005/2009, and between 2009 and years 2006. K concentrations indicated highly significant differences between years for 5/15 pairwise comparisons between 2009 and all other years. Conversely, both Sr and Ba indicated very few pairwise differences (2/15 respectively), with only 2007 significantly different between 2004/2009 for Sr and 2005 and 2009/2010 for Ba respectively (Table 3.19.).







(Figure 3.5. and caption are continued overleaf)

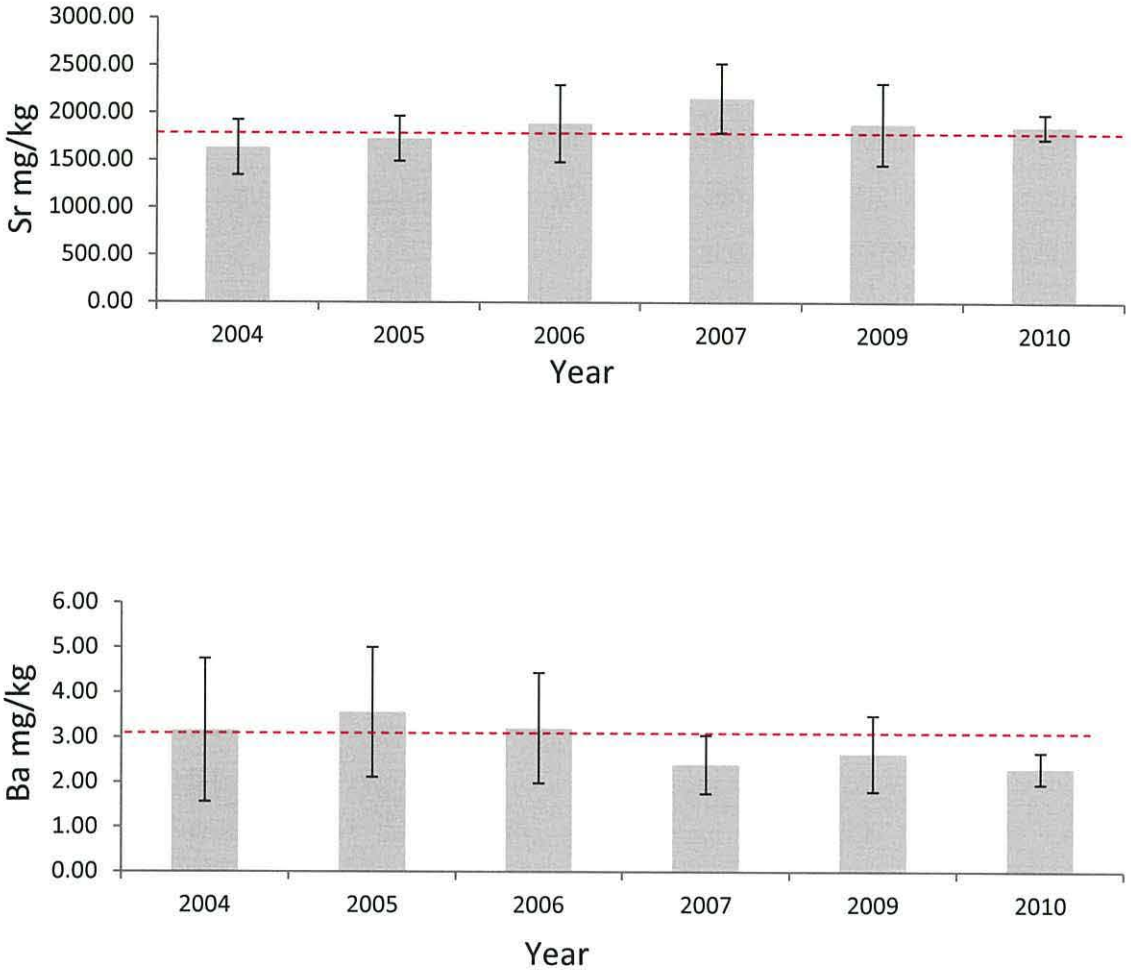


Figure 3.5. Mean (± 1 standard deviation) elemental concentrations (mg/kg) for Na, Mg, K, Sr and Ba in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanddona for the years 2004-2010 (excluding 2008). The dashed line indicates the long term average value calculated from the combined data set.

Mean concentrations of otolith Na, K, Mg, Sr and Ba are presented in Figure 3.6. for each month where sampling took place for each of the six years (2004-2010). Variations in the mean elemental concentrations were apparent for Na, Mg, Sr and Ba between each sampling month and their respective year (Figure 3.6.) indicating possible intra-annual elemental differences. Elemental differences between months within years (intra-annual) and their possible effect observed on the temporal (inter-annual) variability were assessed using ANOVA.

As previously outlined in section 3.5.2., sampling consistency for the site at Llanddona between each year varied with different months sampled in each year (see Figure 3.6.). Due to the sampling inconsistency between years (sampling months varied between all years see Figure 3.6.) and the possible inter-annual effects (*i.e.* sample month and year) of sampling at different periods within years, each month was assessed separately using ANOVA to address inter-annual changes in elemental concentrations between years containing the same month (Table 3.20.). Significant differences in elemental compositions were observed for the sample months of March (K; 2006-2007), July (Sr and Ba; 2004-2005), December (Mg and K; 2005-2006), with highly significant differences observed for the month of August (K; 2005 and 2009 $P < 0.001$). However, no significant differences in elemental concentration were observed for the five elements Na, Mg, K, Sr and Ba for the month of June (comparing years 2004-2005), September (2004-2006) and October (2006 and 2010) ($P > 0.05$ see Table 3.20.).

Table 3.17. Inter-annual differences in Log_{10} transformed Mg:Ca ratio concentrations in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanddona for the years 2004-2010 (excluding 2008) assessed using the non-parametric Kruskal-Wallis test.

Mg	n	DF	Median	Average Rank	Z value	P
Year						
2004	18		0.000119	60.9	2.56	< 0.001
2005	15		0.000122	64.1	2.79	
2006	19		0.000108	37.6	-1.63	
2007	7		0.000104	21.0	-2.63	
2009	13		0.000122	63.0	2.40	
2010	20		0.000105	27.0	-3.70	
Overall	92	5		46.5		

Table 3.18. Pairwise comparisons assessed using Mann-Whitney U test of Log_{10} transformed Mg:Ca ratio concentrations in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanddona for the years 2004-2010 (excluding 2008). Significant differences are highlighted in **bold** and are at the α 0.05 level.

						95% C.I.		
Year	**Median**	**Year**	**n**	**Median**	**W**	**Lower**	**Upper**	**P**
2004	0.00012		18					
		2005	15	0.00012	303.0	-0.00001	0.00003	0.928
		2006	19	0.00011	426.0	0.00000	0.00005	**0.011**
		2007	7	0.00010	287.0	0.00001	0.00008	**0.002**
		2009	13	0.00012	291.0	-0.00001	0.00004	0.920
		2010	20	0.00010	474.0	0.00001	0.00005	**0.0003**
2005	0.00012	2006	19	0.00011	339.0	0.00000	0.00003	**0.008**
		2007	7	0.00010	224.0	0.00001	0.00004	**0.0003**
		2009	13	0.00012	224.0	-0.00001	0.00001	0.782
		2010	20	0.00010	396.0	0.00001	0.00003	**< 0.001**
2006	0.00011	2007	7	0.00010	277.0	-0.00000	0.00002	0.248
		2009	13	0.00012	247.0	-0.00002	0.00000	**0.011**
		2010	20	0.00010	418.0	-0.00000	0.00001	0.292
2007	0.00010	2009	13	0.00012	28.0	-0.00003	-0.00001	**0.0004**
		2010	20	0.00010	90.0	-0.00001	0.00001	0.678
2009	0.00012	2010	20	0.00010	333.0	0.00001	0.00003	**< 0.001**

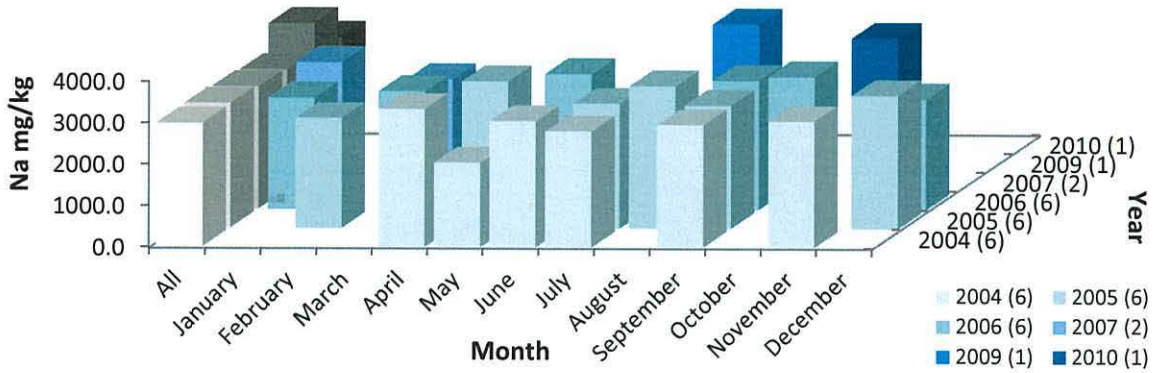
Table 3.19. Summary of the *post-hoc* pairwise comparisons (using a Bonferroni test) comparing the Log_{10} element:Ca ratios of Na, K, Sr and Ba in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanddona between the years 2004-2010 (excluding 2008). Significant differences at the α 0.05 level are indicated in **bold**.

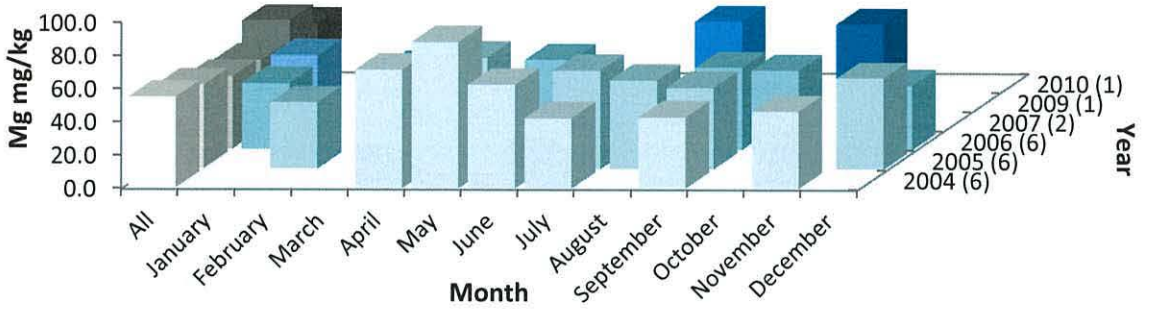
Log_{10} Na	2004	2005	2006	2007	2009	2010
2010	**0.005**	**0.008**	1.000	1.000	**0.002**	
2009	1.000	1.000	**0.026**	**0.003**		
2007	**0.009**	**0.047**	1.000			
2006	0.096	0.557				
2005	1.000					
2004						

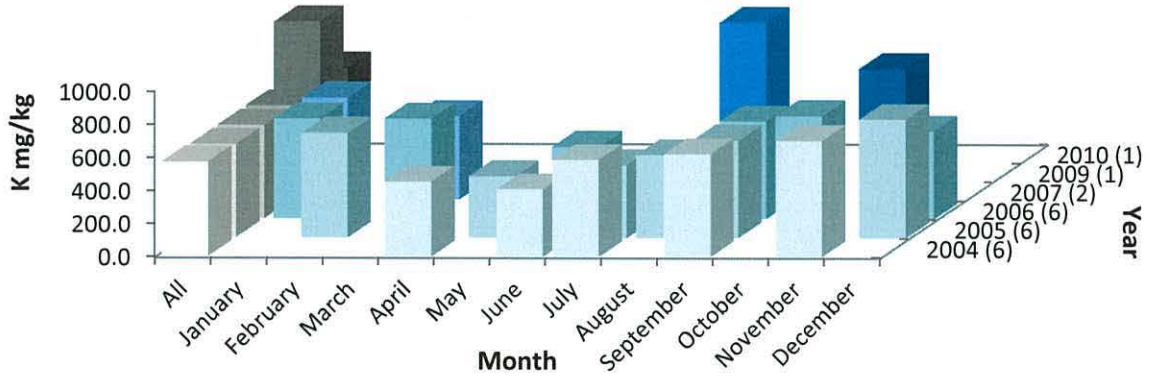
Log_{10} K	2004	2005	2006	2007	2009	2010
2010	0.729	1.000	1.000	1.000	**< 0.001**	
2009	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**		
2007	0.216	1.000	1.000			
2006	0.133	1.000				
2005	1.000					
2004						

Log_{10} Sr	2004	2005	2006	2007	2009	2010
2010	0.249	1.000	1.000	1.000	0.171	
2009	1.000	1.000	0.605	**0.011**		
2007	**0.015**	0.184	0.689			
2006	0.928	1.000				
2005	1.000					
2004						

Log_{10} Ba	2004	2005	2006	2007	2009	2010
2010	0.444	**0.017**	0.275	1.000	1.000	
2009	0.578	**0.034**	0.396	1.000		
2007	1.000	0.117	0.793			
2006	1.000	1.000				
2005	1.000					
2004						







(Figure 3.6. and caption are continued overleaf)

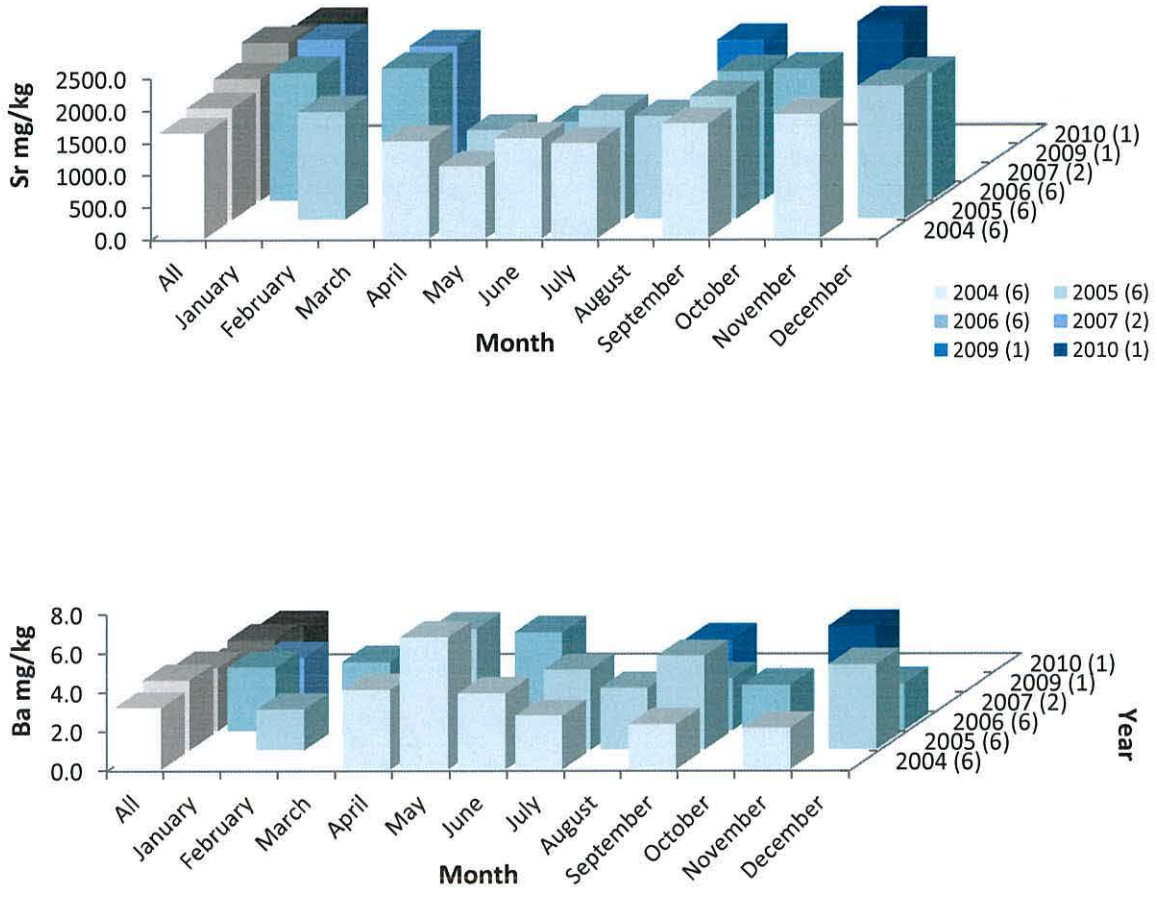


Figure 3.6. Mean elemental concentrations (mg/kg) for Na, Mg, K, Sr and Ba in the sagittal otoliths of juvenile plaice (*Pleuronectes platessa*) from Llanddona for the years 2004-2010 (excluding 2008; see table 3.1. for juvenile plaice sample numbers for each year). Total mean concentrations for the pooled months for each year are described by **All**. Number of months per year are in parenthesis.

Table 3.20. Results of ANOVA analysis to examine otolith element:Ca ratios in the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) from Llanddona caught in the same month but in different sampling years. Significant differences at the α 0.05 level are indicated in **bold**.

Month (Year)	n	Na		Mg		K		Sr		Ba	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
March (2006-2007)	9	1.38	0.279	0.26	0.625	6.68	**0.036**	0.15	0.706	2.31	0.173
June (2004-2005)	5	0.01	0.942	4.86	0.115	0.00	0.969	7.72	0.069	1.00	0.392
July (2004-2005)	7	0.14	0.727	2.13	0.204	3.71	0.112	9.09	**0.030**	9.62	**0.027**
August (2005,2009)	15	3.75	0.075	1.99	0.181	87.59	**<0.001**	0.36	0.557	3.68	0.077
September (2004-2006)	6	0.72	0.556	5.84	0.092	0.43	0.683	2.09	0.271	7.77	0.065
October (2006,2010)	23	0.38	0.543	0.58	0.453	0.10	0.751	0.12	0.736	0.85	0.367
December (2005-2006)	6	0.34	0.589	27.74	**0.006**	8.49	**0.043**	2.61	0.181	7.20	0.055

The problems of inter-annual variability due to possible sampling inconsistency (*i.e.* sampling different months between years) and to increase the range of months used to assess season between years, months were pooled into their respective seasons (see Table 3.9.).

Using ANOVAs run across years comparing season, significant differences were observed in otolith elemental concentrations of Na ($F_{5, 91} = 4.42$; $P = 0.006$), K ($F_{5, 87} = 5.50$; $P = 0.002$), Sr ($F_{5, 91} = 8.89$; $P < 0.001$) and Ba ($F_{5, 89} = 5.64$; $P = 0.001$). K showed significant differences with season and monthly analysis with winter (March and December) indicating changes in chemical composition (Table 3.21.), with highly significant differences observed for season (summer) and month (August) ($P < 0.001$). Both Sr and Ba showed significant elemental changes between season (summer) and month (July). However, significant differences were observed between summer and autumn for Na, with significant effects of season observed for the period of summer for Sr and Ba (Table 3.21.).

Table 3.21. Summary of the *post-hoc* pairwise comparisons (using Bonferroni tests) comparing the elemental concentrations of the Log_{10} transformed element:Ca ratios for Na, K, Sr and Ba between seasons (all years combined) at the nursery ground at Llanddona. Significant differences at the α 0.05 level are indicated in **bold**.

Na	Spring	Summer	Autumn	Winter
Winter	0.276	0.055	1.000	
Autumn	0.207	**0.030**		
Summer	1.000			
Spring				

K	Spring	Summer	Autumn	Winter
Winter	0.025	**0.003**	0.677	
Autumn	1.000	0.211		
Summer	1.000			
Spring				

Sr	Spring	Summer	Autumn	Winter
Winter	1.000	**0.0001**	1.000	
Autumn	1.000	**0.0005**		
Summer	**0.002**			
Spring				

Ba	Spring	Summer	Autumn	Winter
Winter	0.063	**0.031**	1.000	
Autumn	0.061	**0.020**		
Summer	1.000			
Spring				

Using a Pearson's product-moment correlation to assess possible effects of salinity and elemental concentration in a given year, the results indicated no significant correlation between possible freshwater influence and any changes in the elemental concentrations of Na, Mg, K, Sr, and Ba for the years 2004-2010 for the nursery ground at Llanddona (all $P > 0.05$ see Table 3.22.).

Table 3.22. Effects of salinity (river influx) and element:Ca ratios ($x10^{-3}$) of Na, Mg, K, Sr and Ba (using Pearson's correlation) for each of the years 2004-2010 (excluding 2008) at the nursery ground at Llanddona. Elements and salinity are shown as mean ($\bar{x}$) values for each year.

Element	2004	2005	2006	2007	2009	2010	r	*P*
Na	8.056	7.927	7.453	7.028	8.192	7.144	-0.592	0.216
Mg	0.153	0.133	0.111	0.102	0.125	0.105	-0.659	0.155
K	1.616	1.532	1.422	1.370	2.221	1.048	-0.145	0.784
Sr	4.379	4.579	4.793	5.261	4.324	4.824	0.342	0.507
Ba	0.0082	0.0093	0.0083	0.0059	0.0060	0.0059	-0.617	0.192
$\bar{x}$ **Salinity**	32.7	32.9	32.9	33.1	33.0	33.9	-	-

Monthly analysis within year for the elements Na, Mg, K, Sr and Ba were assessed using MANOVA to measure any differences within the chemical concentrations between months sampled within the years 2004-2007. Results indicated significant differences in elemental concentrations of one or more of the five elements Na, Mg, K, Sr and Ba between months and between seasons within the years 2004-2006 ($P < 0.05$, see Table 3.23.). However, the year 2007 indicated no significant differences in the elemental concentrations between months or between seasons ($P > 0.05$, see Table 3.23.).

Table 3.23. Results of MANOVA analysis to examine otolith element:Ca ratios in the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) from Llanddona caught in the same year but in different sampling months and seasons. Significant differences at the α 0.05 level are indicated in **bold**.

Year	Analysis	df	F	*P* value
2004	*Month*	20	2.603	**0.026**
	Season	10	5.974	**0.001**
2005	*Month*	25	2.814	**0.018**
	Season	15	3.590	**0.008**
2006	*Month*	25	3.474	**< 0.001**
	Season	15	7.271	**< 0.001**
2007	*Month*	5	30.433	0.137
	Season	5	30.433	0.137

df. Degrees of freedom.

Table 3.24. Results of ANOVA analysis to examine otolith element:Ca ratios in the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) from Llanddona caught in the same year but in different sampling months and seasons. Significant differences at the α 0.05 level are indicated in **bold**.

Year	Analysis	df	F	*P* value
Na				
2004	*Month*	13	15.09	**0.001**
	Season	13	16.45	**< 0.001**
2005	*Month*	13	5.93	**0.014**
	Season	13	5.00	**0.023**
2006	*Month*	18	4.23	**0.017**
	Season	18	5.52	**0.009**
2007	*Month*	6	23.20	**0.005**
	Season	6	23.20	**0.005**

df. Degrees of freedom.

Table 3.24. Continued.

Year	Analysis	df	F	*P* value
Mg				
2004	*Month*	13	8.29	**0.004**
	Season	13	8.75	**0.005**
2005	*Month*	13	4.42	**0.032**
	Season	13	15.56	**< 0.001**
2006	*Month*	18	5.74	**0.005**
	Season	18	8.68	**0.001**
2007	*Month*	6	3.78	0.109
	Season	6	3.78	0.109
K				
2004	*Month*	13	1.29	0.343
	Season	13	2.42	0.135
2005	*Month*	13	4.42	**0.032**
	Season	13	7.31	**0.007**
2006	*Month*	18	3.24	**0.041**
	Season	18	5.98	**0.007**
2007	*Month*	6	13.83	**0.014**
	Season	6	3.78	0.109
Sr				
2004	*Month*	13	2.49	0.117
	Season	13	2.85	**0.033**
2005	*Month*	13	2.18	0.156
	Season	13	3.37	0.063
2006	*Month*	18	14.59	**< 0.001**
	Season	18	24.99	**< 0.001**
2007	*Month*	6	0.08	0.790
	Season	6	0.08	0.790

df. Degrees of freedom.

Table 3.24. Continued.

Year	Analysis	df	F	*P* value
Ba				
2004	*Month*	13	1.50	0.281
	Season	13	2.85	0.101
2005	*Month*	13	4.47	**0.031**
	Season	13	3.92	**0.044**
2006	*Month*	18	2.71	0.068
	Season	18	4.72	**0.016**
2007	*Month*	6	1.55	0.268
	Season	6	1.55	0.268

df. Degrees of freedom.

Individual ANOVAs indicated significant ($P < 0.05$) or highly significant ($P < 0.001$) intra-annual differences in otolith elemental concentrations for each of the five elements Na, Mg, K, Sr and Ba when looking at months within year (See Table 3.24.). Similarly, ANOVAs run between seasons within years also indicated significant ($P < 0.05$) or highly significant ($P < 0.001$) intra-annual differences in the elemental concentrations for Na, Mg, K, Sr and Ba (see Table 3.24.). Multiple pairwise comparisons (Bonferroni test) significant ($P < 0.05$) differences between months within years were observed for the element Na when comparing the month of April (2004) and the months July, September and November (see Table 3.25.). Similarly, May (2005) and the month December, January (2006 and 2007) and the months June and March respectively (Table 3.25.). Mg indicated both significant ($P < 0.05$) and highly significant ($P < 0.001$) differences in elemental concentrations for 7/15 pairwise comparisons between months and the year 2004. Similarly, significant differences between 4/15 pairwise comparisons between months for the year 2005. The elements K and Sr indicated significant differences between 1/15 and 4/15 pairwise comparisons between months and the year 2006 respectively. In contrast the element Ba was the most stable; with no change in the elemental concentrations between months and each of the years 2004-2007 (see Table 3.25.).

Multiple pairwise comparisons between seasons and years indicated significant ($P < 0.05$) or highly significant differences ($P < 0.001$) between Na (4/16), Mg (7/16) and K (6/16). However, Sr showed highly significant differences ($P < 0.001$) for one year (2006) between summer and the remaining seasons (Table 3.26.). Similarly, Ba showed only one season autumn was significantly different to summer in 2006 (Table 3.26.).

Table 3.25. Summary of the Intra-annual *post-hoc* pairwise comparisons (using Bonferroni tests) comparing the elemental concentrations of the element:Ca ratios for Na, Mg, K, Sr and Ba between months within years at the nursery ground at Llanddona. Significant differences at the α 0.05 level are indicated in **bold**.

Na		Month								
Year		**Mar**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	Apr		1.0000	1.0000	**0.0464**		**0.0328**		**0.0013**	
	May			1.0000	1.0000		0.6745		0.1702	
	June				1.0000		0.7225		0.1018	
	July						1.0000		1.0000	
	Sept								1.0000	
2005	Feb		0.1067		1.0000	0.3000	1.0000			1.0000
	May				0.3547	1.0000	0.1538			**0.0312**
	July					1.0000	1.0000			1.0000
	Aug						0.4871			0.0617
	Sept									1.0000
2006	Jan	1.0000		**0.0497**			0.4141	1.0000		1.0000
	Mar			0.0404			1.0000	1.0000		1.0000
	June						1.0000	0.3306		0.1136
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	**0.0048**								

Mg		Month								
Year		**Mar**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	Apr		**0.0012**	1.0000	**0.0352**		0.0636		**0.0121**	
	May			**0.0010**	**0.0000**		**0.0001**		**0.0000**	
	June				0.6225		0.5955		0.3022	
	July						1.0000		1.0000	
	Sept								1.0000	
2005	Feb		**0.0022**		1.0000	0.1361	1.0000			1.0000
	May				**0.0193**	0.0900	**0.0193**			**0.0082**
	July					1.0000	1.0000			1.0000
	Aug						1.0000			0.8148
	Sept									1.0000
2006	Jan	1.0000		0.0941			0.7090	1.0000		1.0000
	Mar			**0.0055**			0.2189	1.0000		1.0000
	June						1.0000	0.1726		**0.0499**
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.1094								

Table 3.25. Continued

K		Month								
Year		**Mar**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	Apr			1.0000	1.0000		1.0000		0.6981	
	May									
	June				1.0000		1.0000		1.0000	
	July						1.0000		1.0000	
	Sept								1.0000	
2005	Feb		0.0668		0.3877	0.2945	1.0000			1.0000
	May				1.0000	1.0000	0.5804			0.4103
	July					1.0000	1.0000			1.0000
	Aug						1.0000			1.0000
	Sept									1.0000
2006	Jan	1.0000		0.9164			1.0000	1.0000		1.0000
	Mar			**0.0316**			1.0000	1.0000		1.0000
	June						0.1996	0.2700		0.1574
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	**0.0137**								

Sr		Month								
Year		**Mar**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	Apr		0.4159	1.0000	1.0000		1.0000		0.1011	
	May			1.0000	0.5434		1.0000		1.0000	
	June				1.0000		1.0000		1.0000	
	July						1.0000		0.1590	
	Sept								1.0000	
2005	Feb		0.4683		1.0000	0.9059	1.0000			1.0000
	May				1.0000	1.0000	0.6997			1.0000
	July					1.0000	1.0000			1.0000
	Aug						1.0000			1.0000
	Sept									1.0000
2006	Jan	1.0000		0.0251			1.0000	1.0000		1.0000
	Mar			**0.0000**			1.0000	1.0000		1.0000
	June						**0.0012**	**0.0016**		**0.0001**
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.7896								

Table 3.25. Continued

Ba		Month								
Year		**Mar**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
2004	Apr			1.0000	1.0000		1.0000		0.9708	
	May									
	June				1.0000		1.0000		1.0000	
	July						1.0000		1.0000	
	Sept								1.0000	
2005	Feb		0.0984		0.3729	1.0000	0.1580			0.9183
	May				1.0000	0.7814	1.0000			1.0000
	July					1.0000	1.0000			1.0000
	Aug						1.0000			1.0000
	Sept									1.0000
2006	Jan	1.0000		1.0000			1.0000	1.0000		1.0000
	Mar			1.0000			1.0000	0.9162		1.0000
	June						0.7835	0.0954		0.1947
	Sept							1.0000		1.0000
	Oct									1.0000
2007	Jan	0.2682								

Table 3.26. Summary of the Intra-annual *post-hoc* pairwise comparisons (using Bonferroni tests) comparing the elemental concentrations of the element:Ca ratios for Na, Mg, K, Sr and Ba between seasons within years at the nursery ground at Llanddona. Significant differences at the α 0.05 level are indicated in **bold**.

Na 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	**Autumn**
Winter					**Winter**				
Spring					**Spring**	**0.0456**			
Summer		**0.0170**			**Summer**	0.1936	0.6195		
Autumn		**0.0004**	0.0804		**Autumn**	1.000	0.1293	0.9383	
2006	**Winter**	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring	1.000				**Spring**	0.2112			
Summer	0.0503	**0.0078**			Summer				
Autumn	1.000	0.5213	0.2364		Autumn				

Table 3.26. Continued

Mg 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	**Autumn**
Winter					**Winter**				
Spring					**Spring**	**0.0004**			
Summer		**0.0385**			**Summer**	0.0666	**0.0061**		
Autumn		**0.0047**	0.6697		**Autumn**	**0.0048**	1.0000	0.8710	
2006	**Winter**	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring	1.0000				**Spring**	0.1094			
Summer	**0.0172**	**0.0011**			Summer				
Autumn	1.0000	0.1112	0.1358		Autumn				

K 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	**Autumn**
Winter					**Winter**				
Spring					**Spring**	**0.0190**			
Summer		0.2351			**Summer**	**0.0398**	0.6345		
Autumn		0.1971	1.0000		**Autumn**	1.0000	0.1747	1.0000	
2006	**Winter**	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring	1.0000				**Spring**	**0.0137**			
Summer	**0.0378**	**0.0058**			Summer				
Autumn	1.0000	1.0000	**0.0215**		Autumn				

Sr 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	**Autumn**
Winter					**Winter**				
Spring					**Spring**	0.1721			
Summer		1.0000			**Summer**	0.4319	1.0000		
Autumn		0.1156	0.0565		**Autumn**	1.0000	0.2148	0.6836	
2006	**Winter**	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring	1.0000				**Spring**	0.7896			
Summer	**0.0000**	**0.0000**			Summer				
Autumn	1.0000	1.0000	**0.0000**		Autumn				

Table 3.26. Continued

Ba 2004	Winter	**Spring**	**Summer**	**Autumn**	**2005**	**Winter**	**Spring**	**Summer**	**Autumn**
Winter					**Winter**				
Spring					**Spring**	0.1233			
Summer		1.0000			**Summer**	0.7634	0.7257		
Autumn		0.1814	0.2645		**Autumn**	0.2303	1.0000	1.0000	
2006	**Winter**	**Spring**	**Summer**	**Autumn**	**2007**	**Winter**	**Spring**	Summer	Autumn
Winter					**Winter**				
Spring	0.7910				**Spring**	0.2682			
Summer	0.0523	0.4710			Summer				
Autumn	1.0000	0.3716	**0.0237**		Autumn				

Linear discriminant function analysis (LDFA) was used to look at temporal stability of the elemental concentrations of Na, K, Sr and Ba in juvenile plaice sagittal otoliths from Llanddona between 2004-2010. If the chemistry is temporally stable, poor percentage classification to year would be predicted.

The percentage correct classification to year based on the sagittal otolith chemistry was 52.2% with a reduced overall percentage cross validation classification of 42.5% (Table 3.27.). The low percentage correct classification rate was more pronounced for the years 2006 (26.3%) and 2005 (42.9%). Only the years 2007 and 2009 indicated a correct classification rate above 80% (85.7% and 100% respectively), with 2004 and 2010 both having correct classification rates of $\geq$ 50% (50% and 55% respectively Table 3.27.). The percentage correct classification rates were reduced further using cross validation counts for all years (42.5%), with the years 2005 and 2006 indicating classification rates below 20% (14.3% and 15.8% respectively). Only one year (2009) indicated no change in percentage correct classification rate for both the original and cross validation analysis (100%) and was the most distinct year in terms of its chemistry.

Using principal component analysis (PCA), multi-elemental analysis of the four elements Na, K, Sr and Ba indicated relatively poor separation (Figure 3.7.) for years 2004-2007. In contrast, the years 2009-2010 indicated high separation between earlier years and themselves (Figure 3.7.). Total variance was explained by the element Sr (40.2%) in PC1 with Ba explaining the variance observed in PC2 (32.5%).

Table 3.27. Linear discriminant function analysis (LDFA) original classification (top of table) and cross validation classification, leave one out method (bottom table) of juvenile plaice (*Pleuronectes platessa*) collected from Llanddona during 2004-2010 (excluding 2008) using element:Ca ratios of Na and Sr. Numbers in **bold** indicate percentage of correctly classified fish to their sample year. Total n = number of individuals analysed with their total accumulated percentage of correctly classified fish in parenthesis.

	Predicted nursery ground Llanddona						
	2004	**2005**	**2006**	**2007**	**2009**	**2010**	**Total *n***
Original Count							
2004	**7 (50%)**	2	2		1	2	14
2005	3	**6 (42.9%)**	4		1		14
2006	2	4	**5 (26.3%)**	4		4	19
2007			1	**6 (85.7%)**			7
2009					**13 (100%)**		13
2010	3	1	1	4		**11 (55%)**	20
							52.2%
Cross Validation Count							
2004	**5(35.7%)**	2	2		3	2	14
2005	5	**2 (14.3%)**	6		1		14
2006	3	5	**3 (15.8%)**	4		4	19
2007			1	**4 (57.1%)**		2	7
2009					**13 (100%)**		13
2010	3	1	2	4		**10 (50%)**	20
							42.5%

*Light and dark panels indicate adjacent years to which fish were incorrectly classified from their original sample year when using LDFA.

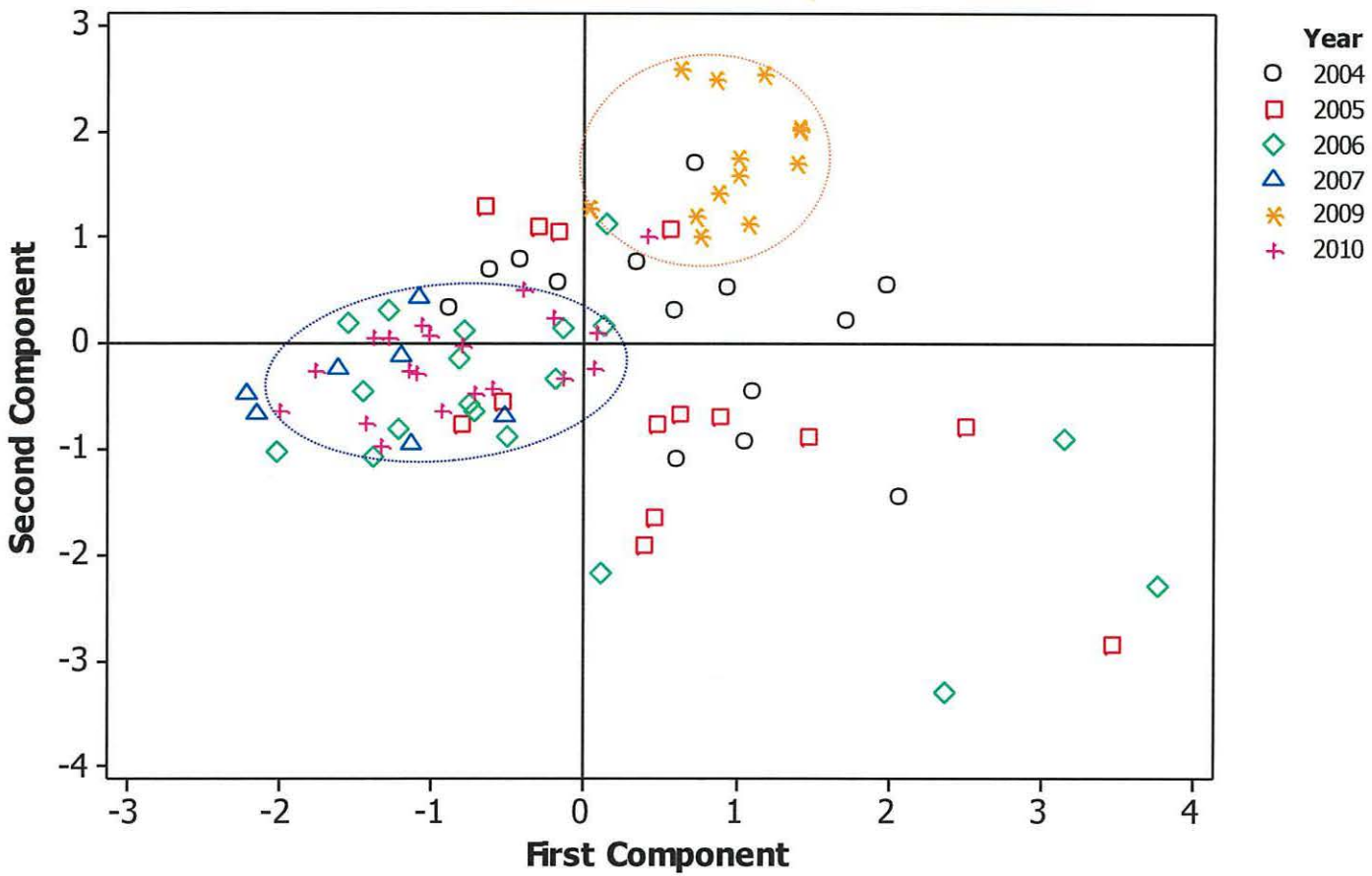


Figure 3.7. PCA displaying year class from the sagittal otolith of juvenile plaice (*Pleuronectes platessa*) collected from Llanddona during 2004-2010 (excluding 2008) using element:Ca ratios of Na, K, Sr and Ba. Clustering used to allow year class overlap to be observed.

3.6. Discussion

In the present study, variations in the elemental concentrations (Na, Mg, K, Sr and Ba) of sagittal otoliths of juvenile plaice were observed on both an inter-annual (between years) and intra-annual (between both season and month) scale at both Llanddona and Llanfairfechan for the time period 2004-2010. Significant inter-annual variations in the chemical compositions of plaice otoliths were observed for Mg (2007-2010), Sr (2005-2010) and Na (2009-2010) at Llanfairfechan, and for Na (2007-2010), K (2007-2010), Sr (2007-2009) and Ba (2009-2010) at Llanddona. However, of the two sites Llandonna indicated greater variability when looking at the intra-annual scale for the present study.

Previous temporal stability studies have shown inter-annual variation in the chemistry of otoliths extracted from fish sampled from either rivers, estuaries or coastal regions (Rooker *et al.*, 2001; Kerr *et al.*, 2007; Walther *et al.*, 2008; Tanner *et al.*, 2012) when looking at one or more of the following elements: Li, Na, K, Mg, Mn, Sr and Ba (see Table 3.28. and review Table 4 in Gillanders, 2002). Furthermore, other studies have observed significant variation in trace element concentrations in otoliths over a 2-3 year interval: for Li, Mg, Cu, Zn, Sr and Ba (Milton *et al.*, 1997); Mn and Ba (Dove and Kingsford, 1998); Ba (Patterson *et al.*, 1999); Mn, Sr and Ba (Gillanders and Kingsford, 2000) (See Table 3.28.). Milton *et al.* (1997) have shown elemental concentrations of Mg, Cu, Zn, Sr and Ba to be elevated (with Li depleted) in 1995 in tropical shad (*Tenualosa toli*) compared to the concentrations a year earlier. Similarly, Gillanders and Kingsford (2000) observed inter-annual variability for Mn, Sr and Ba concentrations in juvenile trumpeter (*Pelatus sexlineatus*) otoliths. Sampling over a period of two successive years (1998-1999) and at two to five sites within each of seven estuaries along the east coast of Australia, Gillanders and Kingsford (2000) observed variability within the elemental composition of the otoliths of *P. sexlineatus* not only within and amongst the estuaries but also between the two years of sampling. Further studies have observed temporal instability between years (Edmonds *et al.*, 1992; Edmonds *et al.*, 1995; Dove and Kingsford, 1998; Patterson *et al.*, 1999; Campana, 2000; Rooker *et al.*, 2001) and within years (Kalish 1989; Kalish, 1991; Edmonds *et al.*, 1995; Thorrold *et al.*, 1998*b*; Thorrold and Shuttleworth, 2000; Swearer *et al.*, 2003).

Table 3.28. Review table of studies examining the temporal variation observed in the elemental chemistry of fish otoliths. Sites: **C**: coastal, **E**: estuarine, **L:** lake, **Re**: reef, **Ri**: river, **S**: shelf. (Adapted from Gillanders, 2002; Elsdon *et al.*, 2008)

Temporal Scale	N° of Sites	Elements Analysed	Significant Differences	Environment	Source
Within Year					
2 collections in 6 months	1	Mn, Sr, Ba	Mn (not Ba & Sr)	Ri/E	Thorrold & Shuttleworth, 2000
Aug. & Oct. in 1 Yr.	1	Mg, K, Mn, Sr, Ba	Among sampling occasions	Ri	Thorrold *et al.*, 1998*b*
4 collections, 3 month intervals in 1 Yr.	1	Na, Mg, P, S, Sr	Seasonal variation within Canonical plot		Edmonds *et al.*, 1995
8 collections in 1 Yr.	1	Na, K, S, Sr	All elements Seasonal variation	C	Kalish 1991
85 collections, paired samples 1-2 hours apart	1	Mn	Mn among samples collected hours apart	Ri	See Elsdon *et al.*, 2008
2 collections 2 tides.	1 in 2 estuaries	Ca, Mn, Sr, Ba	Ca, Mn, Sr, Ba among tides 1 site	E	Elsdon & Gillanders, 2006*a*
16 collections nested design (2 x seasons, months, wks., days)	1 in each of 3 estuaries	Ca, Mn, Sr, Ba	Mn, Sr –season. Ca, Mn, Sr weeks. Ca, Mn, Sr, Ba Days.	E	Elsdon & Gillanders, 2006*b*
Weekly collection over 100 days	1	Mn	Among times (see table in paper)	C	Grotti *et al.*, 2001
Nested tidal cycle (Flood, slack, ebb)	1	Mn (also Al, Fe, Cu, Cr, Zn, Pb)	Mn hours	E	Hatje, 2003
6 collections 2/month for 3 months	6 in 5 estuaries	Mg, Mn, Sr, Ba	Sr month (not Mg, Mn, Ba)		Dorval & Jones, 2005
Monthly collections in 1 Yr.	1	Na, K, S, Sr	Variation all elements within Yr.	C & S	Kalish 1989
Among Years					
3 collections over 3 Yrs.	1	Li, Mg, Mn, Sr, Ba	Mg, Mn, Ba inter-annual trends		Rooker *et al.*, 2001
Aug. & Sept. in 3 Yrs.	4	Ca, Sr	Between sites & Yrs.	Ri/E	Kerr *et al.*, 2007
2 collections over 2 Yrs. & 3 Yrs.	3	Mg, Mn, Sr, Ba	Sr between Yrs. (no analyses on other elements)	Ri/E	Walther *et al.*, 2008
Collection in 1 Yr.	9	Mg, Mn, Sr, Ba	As Above	Ri/E	Walther *et al.*, 2008
2 collections pooled/Yr. over 2-3 Yrs.	4	Li, Mg, Mn, Sr, Ba	Relatively similar	C*	Campana *et al.*, 2000
~5 collections over 13 Yrs.	4	Li, Mg, Sr, Ba	Little change over 2 Yrs. More substantive for some elements & locations after 4-13 Yrs.	C*	Campana *et al.*, 2000
2 collections ~1 Yr. apart	2-5 in each of 7 estuaries	Mn, Sr, Ba	Temporal effects some &/or All estuaries	E	Gillanders & Kingsford, 2000
2 collections in summer 2 Yrs.	1	Zn, Sr, Ba, Pb	Ba	C	Patterson *et al.*, 1999
2 collections ~1 Yr. apart	2 in each of 6 locations	Mg, Al, Ti, Mn, Co, Cu, Zn, Rb, Sr, Ba, Hg, Pb	Mn, Ba	Re	Dove & Kingsford, 1998
2 collections 1 Yr. apart	5	Li, Na, Mg, Cu, Zn, Sr, Ba	Between Yrs. all elements (not Na)	E	Milton *et al.*, 1997
2 collections 2 Yrs. apart	3	Na, Mg, P, S, K, Sr	S, P Temporal variation (no stats)		Edmonds *et al.*, 1995
3 collections 3 Yrs.	3	Na, Mg, P, S, Sr	Temporal variation in CV-Plot elements not mentioned		Edmonds *et al.*, 1995
2 collections ~2 Yrs. apart	3	B, Na, Mg, K, Mn, Sr	As above	C	Edmonds *et al.*, 1992
1 collection 2 Yrs. synoptic survey	25	Na, Ca, Mn, Sr, Ba	Element:Ca ratio geographically consistent wet & dry Yr.	L/Ri	Limburg & Siegel, 2006

* Both off-shore and in-shore sites. Yr.(s) - year(s), Wks. - weeks.

The study with the longest (but non-consecutive) time series of data is that reported by Campana *et al.* (2000) for Atlantic cod *Gadus morhua* in the Gulf of St. Lawrence. They found significant variation in the elemental concentrations of Li, Mg and Ba both between sites and within sites over time (4-13 years). However, their studies over that temporal scale were not conducted using a continuous data set (4 year periods between sampling, see Figure 5 in Campana *et al*, 2000). Furthermore, significant variation within the chemistry of the otoliths over 2 to 3 year intervals has been observed in several other studies (Patterson *et al.*, 1999; Gillanders and Kingsford, 2000; Rooker *et al.*, 2001). The present study is one of the longest time series (6 years) looking into temporal stability presenting data for two geographically-close plaice nursery grounds (Llanddona and Llanfairfechan) for the time period 2004-2010. The results for the present study were similar to the observations reported by Campana *et al.* (2000) for their long-term analysis (4-13 years), with inter-annual variability observed for both Mg and Sr during the 6 year analysis for the nursery grounds at Llanfairfechan and Llanddona. However, in contrast to the results of Campana *et al.* (2000) and those recorded at Llanfairfechan, the elemental concentrations of Na, K, Sr and Ba were found to be relatively stable at Llanddona between all years during the 6 year analysis in the present study. When considered over short time scales (*i.e.* a 2 year period) the results of Campana *et al*, (2000) and the present study on plaice have shown that some temporal stability could be observed relative to the life cycle of the fish. Campana *et al.* (2000) found the concentrations of most of the elements analysed (Li, Mg, Sr and Ba) varied relatively little over a shorter 2 year period. The results for the present study indicated some stability in Mg (Llanfairfechan) and Na and Sr (Llanddona) over a 3 year period (2004-2006), increasing to 4 years (2004-2007) for Na and Ba at Llanfairfechan and K and Ba at Llanddona. It is interesting to note that these were the years during which the most monthly samples were obtained providing a more reliable average annual elemental signature.

The variability observed in the elemental concentrations measured for the latter years 2007-2010 compared to 2004-2006 for the elements Na, Mg and Ba at Llanfairfechan and Na, K, Sr and Ba at Llanddona may be the result of sample sizes (*i.e.* small sample number for 2007 at both sites) or sampling frequency (only 1 month sampled during 2009 and 2010 at both sites). Samples of 1-group plaice were originally selected from the retained plaice samples (where 1-group plaice were collected in that month / year) from a previous Ph.D. project where 0-group fish were the target (Al-

Rashada, 2009, unpublished data). 1-group plaice collected during the later years (2009-2010) were sampled in a single month: July at Llanfairfechan and August at Llanddona during 2009, with samples collected in October at both sites in 2010.

Inter-annual variations in monthly otolith chemistry were also observed during the present study for both sites when comparing between the same month in different years *e.g.* Sr over a 3 year interval (2005-2007) for March at Llanfairfechan and K (March) and Mg and K (December) for 2 year intervals (2006-2007 and 2005-2006 respectively) at Llanddona. Seasonal effects on the chemical composition were also observed in the present study with Mg showing temporal variability between the summer and spring, autumn (Llanfairfechan), and significant variations observed in the otolith concentrations of Na, K, Sr and Ba for most of the seasons at Llanddona. Previous studies have also documented significant intra-annual variability in Na, S, K and Sr otolith concentrations for fish caught in a range of habitats (Kalish, 1989, 1991; Edmonds *et al.*, 1995; Thorrold *et al.*, 1998*b*) (see Table 3.28.). Additionally, significant variation in elemental composition has further documented for shorter time periods, *e.g.* among months or seasons, for Mg, Mn, Sr and Ba (Thorrold *et al.*, 1998*b*), Mn (Hamer *et al.*, 2003), Sr and Ba (Patterson and Kingsford, 2005).

The most notable intra-annual variation was observed at Llanddona compared to Llanfairfechan. The intra-annual results for Llanfairfechan, found the elemental concentrations were more stable with little or no variability observed between months within years for each of the five elements Na, Mg, K, Sr and Ba. Similar results were also observed when looking at season and the same suite of elements at Llanfairfechan. However, Llanddona was found to be predominantly the least stable of the two nursery sites, especially for Na, and Mg. One explanation for the observed temporal variability for Na and Mg may be their susceptibility to leaching. Na and Mg have both been described as being more labile (*i.e.* easily leached out of the otolith matrix) compared to elements which substitute for Ca in $CaCO_3$ (*e.g.* Mn, Sr and Ba; Hedges *et al.*, 2004). This may be one possible factor contributing to the changes in their chemistry and one identified by Milton and Chenery (1998) who found significant effects of both storage duration and extraction time on Mg obtained from frozen otoliths. Samples used in the present study were stored frozen between 2-4 years before extraction and may be subject to the same problem observed by Milton and Chenery (1998) (although, see Rooker *et al.*, 2001; Swan *et al.*, 2006 on storage effects on Mg) and not down to possible elemental changes over the inter-annual and intra-annual scale.

The molar concentration of magnesium within sea water has been shown to be around five times that of calcium (Irving, 1926) and varies in proportion within marine sediments (as magnesium carbonate, $MgCO_3$) according to the remains of the organic constituents producing them (Clarke and Wheeler, 1922; Irving, 1926). One mode of possible Mg influx (and possibly the resulting variability observed at the site at Llanddona) may be as a result of the faunal distribution within and around the site. Environmental factors such as the physical processes (*i.e.* wave action and currents) or habitat suitability may play a contributing role by influencing the abundance of molluscs shells at one site compared to another (see Allen and Moore, 1987). Furthermore, higher volumes of shell debris from off-shore populations of bivalves have been observed in the fine sands of Llanddona (Allen and Moore, 1987) and may be contributing to the presence of Mg. This increased shell density, may help explain the significant differences observed in elemental concentrations between months within years and between years at Llanddona.

Another factor that could influence the results obtained may be the effect of temperature on the incorporation of elements into the otolith. This aspect has been studied more than any other variable, with elements incorporated within aragonite structures (*e.g.* otoliths and corals) appearing to fluctuate as a function of temperature (Rooker *et al.*, 2001). Studies on the influence of temperature and salinity have been reported for several elements but most notably for the element Sr (Kalish, 1989; Townsend *et al.*, 1992; Hoff and Fuiman, 1995). Although the effects of temperature were not assessed in the present study, the possible effects of changes of salinity in the present study found no correlation between the years (2004-2010) and elemental concentrations for either nursery grounds. However, the possible influence of freshwater (change in salinity) may be evident between both sites when looking at the element Sr (although the analyses conducted were basic and limited to small sample sizes). Studies have indicated the inter-annual effect of water chemistry on otolith elemental signatures (See Milton *et al.*, 1997; Patterson *et al.*, 1999; Campana *et al.*, 2000; Rooker *et al.*, 2001), with Sr levels being associated with differences in the ambient water chemistry between sites / nursery grounds (Rooker *et al.*, 2001). Furthermore, the element Sr has been positively correlated with changes of salinity in sea water (Ingram and Sloan, 1992; Limburg, 1995).

The site at Llanfairfechan is a marine coastal area on the North Wales coast however, The Afon Llanfairfechan runs directly through Nant y Coed nature reserve and

drains into marsh lands to the south and west and onto the shore of Traeth Lafan. The effects of this freshwater influence and the change in the Sr levels between years at Llanfairfechan may be one of the contributing factors for the results observed at this study site. However, caution must be applied in interpreting the temporal patterns as the data set for the latter years is limited (*i.e.* only one sample month in 2009 and 2010). In contrast, the site at Llanddona appears unaffected by the influence of possible freshwater runoff with Sr concentrations remaining relatively stable for all years with the exception of 2007. This could be as there is no major freshwater input into the area surrounding Llanddona.

Even though freshwater influx from riverine sources near both sites at Llanfairfechan and Llanddona (see Figure 3.4.1.) were not correlated with otolith chemistry (all Pearson's correlations $P > 0.05$) we cannot discount the possible effects of freshwater on the biology of the juvenile plaice and the temporal instability inferred from the ANOVAs (Tables 3.3.5. and 3.3.17.). The drop in salinity and slight increase in rainfall at Llanfairfechan during 2007, combined with the sharp increase observed in salinity during 2010, may be a contributing factor as to why these years indicate significant differences in the elemental concentrations of Na, Mg, Sr and Ba. Similarly, the effects of a decrease in rainfall combined with an increase in salinity for the site at Llanddona may also account for the elemental changes observed for the elements Na and K for the years 2007 and 2009.

The problems that can be associated with the analysis of fish otoliths using conventional microchemistry (*e.g.* solution based or laser ablation applications) are not due to the techniques used to analyse these calcium carbonate structures, but the very environment those fish reside in during their early life stages and for how long (see Elsdon and Gillanders, 2005). Individual fish (depending on the species) may reside in a chosen nursery area / ground from anything from a few months to a few years (Beck *et al.*, 2001, Able, 2005; Vasconcelos *et al.*, 2008). An example of which can be seen for the juvenile plaice (*Pleuronectes platessa*), which indicate both site fidelity and restricted movement patterns when small (see Burrows *et al.*, 2004) spending anything between 1-3 years within their chosen nursery area before migrating offshore to join the adult population (Nash *et al.*, 1994). Therefore the problem of residence time can play a major factor in establishing the elemental concentrations of a specific area / site being incorporated within the otoliths. The time spent by individual fish within these areas would require a residency period long enough (> 20 days, see Elsdon and Gillanders,

2005) for the elements within the surrounding ambient water to be incorporated within the lattice matrix of the otoliths to be detected accurately by the appropriate analytical techniques.

The question of the importance of residency time can, in part, be explained by the pathways and time involved in the incorporation of elements from the surrounding water into the otolith since the otoliths are not in direct contact with the surrounding ambient water. The uptake of trace elements and ions is a multi-stage process controlled not only by physiological (cellular) factors but also by biological factors (*e.g.* concentration gradients) within highly osmoregulated organisms such as fish (see reviews by Campana, 1999; Elsdon and Gillanders, 2005). Elements such as Ca, Sr and Ba and other trace elements within the water must first pass through these boundaries before becoming incorporated within the lattice structure of the otoliths. Changes within the nursery area (*i.e.* environmental changes) may be more or less instantaneous (*e.g.* high rain fall causing a salinity change or anthropogenic activities leading to leaching into the surrounding water course) changing the elemental composition of the surrounding water body. These changes would in essence change the environmental history of that nursery area / site not only spatially but also temporally (Elsdon and Gillanders, 2005; Elsdon and Gillanders, 2006*b*). However, there may be a time lag in the uptake and incorporation of elements into the otolith matrix which may provide a buffer from rapid temporal changes in the surrounding water chemistry as a result of a sudden influx of fresh water. Elsdon and Gillanders (2005) have indicated that for the incorporation of certain elements such as Ca, Mn, Sr, and Ba within the otoliths of juvenile black bream (*Acanthopagrus butcheri*), individuals would need to occupy a given body of water (in this case an estuary) for a period greater than 20 days if any reliable chemical signatures representative of that water body were to be observed in the otolith. However, they also comment that the assumption of residency duration and elemental inclusion in adult fish have not yet been tested for Ca, Mn, Sr, and Ba (Elsdon and Gillanders, 2006*b*). Other factors such as the effects of physiological processes (*i.e.* metabolism and diet) may also influence trace elemental uptake and deposition in otoliths and should also be investigated (see Kalish, 1989, 1991; Gillanders and Kingsford, 2003).

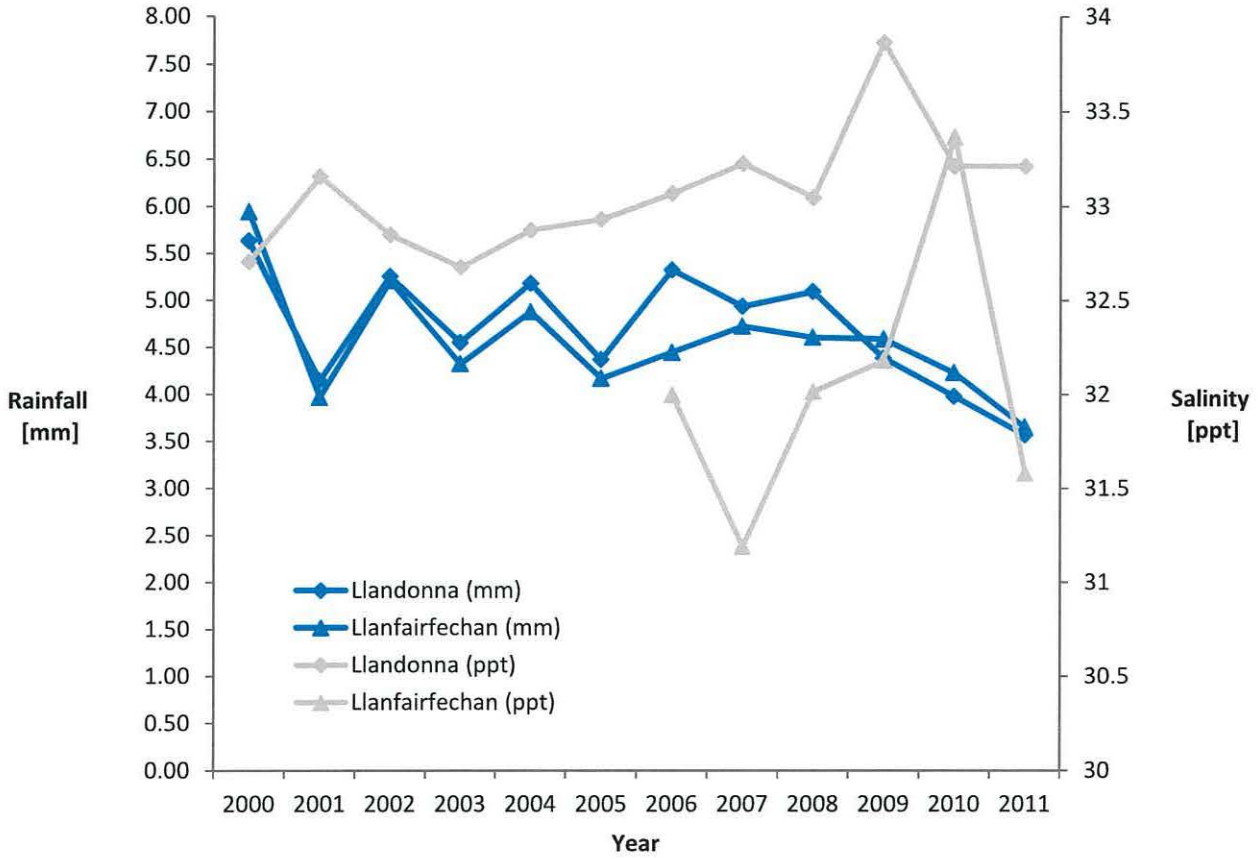


Figure 3.4.1. Plotted rainfall and salinity data for both Llanfairfechan and Llanddona. Rainfall and salinity are shown as mean ($\bar{x}$) values for each year (2000 and 2011). Salinity was only recorded from Llanfairfechan from 2006. Data courtesy of the Natural Resources, Wales

3.6.1 Conclusion

The aim of the present study was to determine whether temporal stability was observed in otolith microchemistry over an extended time period (2004-2010) using juvenile plaice sampled from two nursery grounds in northwest Wales (Llanfairfechan and Llanddona). The results indicated significant inter-annual (*i.e.* between years) and intra-annual (*i.e.* within years, month and season) variation in otolith microchemistry. This is an important result as one assumption in using otolith chemistry to identify the origins of fishes (*e.g.* nursery ground) is that any site-specific chemical tag is stable over time. However, the results must be taken as being indicative rather than conclusive since the study was opportunistic rather than based on a well-designed sampling programme. The otoliths analysed in the present study were derived from fish samples from a previous Ph.D. project retained in the freezer for periods of time ranging from 1 year to 7 years. It is possible that there may have been some effect of storage duration on the measured otolith chemistry (see Chapter 2 section 2.6. for a discussion on storage duration) although the results of Chapter 2 indicate no effect of storage duration after one year. Also, in order to test the hypothesis of temporal stability requires a more robust sampling approach than the opportunistic set of samples analysed in the present study. Sampling using nested designs (more commonly described as "Hierarchal" designs; Underwood, 1997) and nested statistical analysis to interpret the results would provide a more powerful approach to address the question of temporal stability (Elsdon and Gillanders, 2006*b*). Although push netting was conducted from the same general area at both plaice nursery beaches, the actual location was not determined using GPS to ensure the exact location was sampled. In addition sampling inconsistency through netting in different months and over different time periods in each year could also confound the results obtained. The results of the present study suggested that for some elements there was some degree of temporal stability between years: Na, Mg and Ba at Llanfairfechan and Na, K, Sr and Ba at Llanddona. Llanddona showing more stability for Sr and Ba, elements which tend to substitute for Ca within the lattice structure of the otolith by substituting for Ca (Campana, 1999).

In conclusion, to enable a better understanding of the influences affecting otolith chemistry further investigation is required not only of differing species but the environmental effects acting upon those fish and whether we can understand and predict

those changes over temporal scales (Gillanders, 2002; Elsdon and Gillanders, 2008). Predicting these changes may enable scientists to understand how certain elements used in otolith research (*i.e.* Sr and Ba; Elsdon and Gillanders, 2008) are incorporated from the surrounding ambient water by further examining the variability observed within the environment for these elements (geological changes). This may elucidate other methods which could be used to identify fish movement, stock structure and the issues of philopatry (Elsdon and Gillanders, 2008) using temporal analyses and which are necessary prerequisites if we are to use these methods in future fisheries science (Rooker *et al.*, 2001).

Chapter 4

The use of biogeochemical tags to infer movement patterns of adult sea trout and juvenile brown trout (*Salmo trutta* L.) parr

Abstract

Understanding and obtaining information on fish population dynamics and the knowledge of their origins and movement patterns is important in understanding important life history characteristics such as where they go at different stages of their life cycle and why they go. The use of multi-elemental otolith microchemistry is rapidly becoming a useful tool for stock discrimination and the reconstruction of individual fish migratory patterns. Furthermore, these unique spatial geochemical signatures have enabled studies to infer natal origins of fish. The Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca ratios in the sagittal otolith were used as a natural biogeochemical tag to determine natal rivers / sub-region of origin of 665 juvenile brown trout parr (*Salmo trutta*) sampled from 36 main sea trout producing rivers in SW Scotland, NW England, Wales, Isle of Man and the east coast of Ireland which flow into the Irish and Celtic Seas. Using the element:Ca ratios of Mg, Mn, Sr and Ba from trout parr otoliths a biogeochemical baseline was produced and then used to assign "blind" run parr samples (n=39) to their source. Using the now established biogeochemical baseline 219 adult sea trout captured from 5 marine zones within the coastal waters of the Irish Sea were then assigned to their putative sub-region of origin using material ablated from their freshwater period of growth in their sagittal otolith. Significant differences were observed between each of the 36 rivers using cross-validation quadratic discriminant function analysis (CV-QDFA), with an overall classification accuracy of 74% for assigning juvenile trout parr back to their natal rivers, with 66% of trout parr being correctly classified back to region. Using the now established biogeochemical baseline, 69% (27/39) of "blind" run trout parr were identified back to their river of origin. Adult sea trout of unknown freshwater origin were assigned to their putative natal sub-region using the biogeochemical baseline created from the CV-QDFA and their freshwater residency. Assuming that sea trout remain in coastal waters close to their river of origin, the number of adult sea trout caught in a given marine zone that classified back to the adjacent freshwater sub-region was determined: less than 20% of adults were assigned to the adjacent freshwater sub-region. Since classification accuracy of trout parr to river / region was high and indicated the baseline was robust and the allocation of marine caught adult fish to sub-region were low, the results of this study suggest that adult sea trout may be undertaking more extensive marine migrations within the Irish Sea than previously thought.

4.1. Introduction

Many fish species are known to undertake movements between different habitats during their lifetimes and large-scale geographical movements are not uncommon in a range of fish taxa: for example, in elasmobranchs (*e.g.* Bonfil *et al.*, 2005), chondrosteans (*e.g.* Jennings and Zigler, 2000) and teleost fishes (*e.g.* Groot and Margolis, 1991; Hansen and Quinn, 1998; Aarestrup *et al.*, 2009; Garcia Vasquez *et al.*, 2009; Madigan *et al.*, 2012). Tracking these movements has proved problematic and a range of techniques utilising externally applied and natural internal markers have been used to try to determine movement patterns of fishes (see Chapter 1, section 1.2. for a detailed discussion of applied and natural tags). However, despite the difficulties in obtaining this information, knowledge of the origins of fishes and their movement patterns is important in understanding the ecology of a species, *i.e.* where they go at different stages of their lifecycle and why they go there. In addition, understanding the movement patterns of a species at different stages of its life cycle and how different populations of a species may intermix is important in the development of sustainable management strategies for commercially exploited fish species.

Salmonid fishes, of the genus *Salmo,* are known for their highly variable life cycles, whereby individual fishes can adopt different life history strategies with regards to movement patterns (both within freshwater and between freshwater, estuarine and marine environments see Figure 4.1. on life cycle of brown trout), the age / size at which they undertake these movements and the age / size at first reproductive maturity (*e.g.* Elliott, 1994; Marschall *et al.*, 1998; Klemetsen *et al.*, 2003; Cucherousset *et al.*, 2005). Such variability is described as phenotypic variation and a summary of the range of phenotypic variation observed in the Atlantic salmon *Salmo salar* and the brown trout *Salmo trutta* is presented in Table 4.1. As Table 4.1. indicates, both species show considerable variation in patterns of movement and maturity. Historically, greater emphasis and research effort have been focused on the Atlantic salmon compared to the brown trout given its socio-economic importance and for *Salmo trutta*, greater emphasis has focused on the freshwater-resident form rather than the anadromous sea trout which has been described as "overlooked" or "neglected" by some authors (Harris and Milner, 2006). In this chapter, *Salmo trutta* will be used hereafter to refer to the species whilst the terms brown trout and sea trout will be used to refer specifically to the freshwater-resident and sea-run morphs respectively.

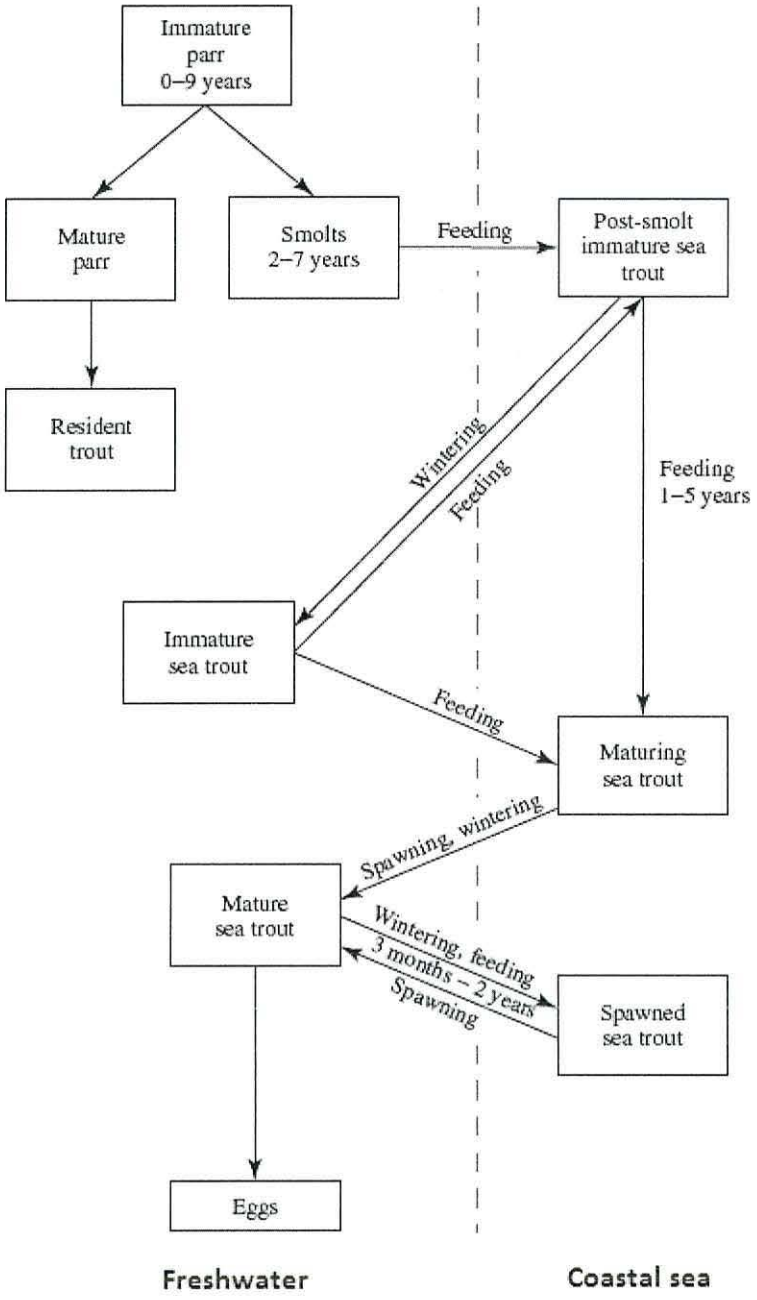


Figure 4.1. Life cycle of anadromous brown trout *Salmo trutta*. showing both resident and migratory forms (taken from Jonsson and Jonsson, 2009).

Table 4.1. A summary of the phenotypic and life history variation observed in fish of the genus *Salmo*: Atlantic salmon *S. Salar* L. and brown trout *S. trutta* L.

Observed Life history variation	Species	Frequency of Occurrence	Comments and References
A) Movement Patterns			
Non-marine migration, completion of lifecycle in freshwater	*S. salar*	Uncommon	Sebago salmon/Ounaniche: Klemetsen *et al.* (2003)
	S. trutta	Common	Brown trout: Jonsson (1985), Elliott (1994), Klemetsen *et al.* (2003)
Variable age/size at migration to sea (smoltification)	*S. salar*	Common	1 – 8 years old, 12-20 cm: Klemetsen *et al.* (2003)
	S. trutta	Common	1 – 4 years old, 9-21 cm: Klemetsen *et al.* (2003)
Migration to estuarine environment without movement into full strength seawater	*S. salar*	Uncommon	Karlsson & Karlström (1994); Klemetsen *et al.* (2003)
	S. trutta	Common?	Slob trout: Klemetsen *et al.* (2003), Etheridge *et al.* (2008)
Migration within freshwater between tributaries and mainstem of river	*S. salar*	Common	Klemetsen *et al.* (2003)
	S. trutta	Common	Cucherouset *et al.* (2003), Klemetsen *et al.* (2003)
Migration between lacustrine and riverine environments in same catchment	*S. salar*	Uncommon	Hutchings (1986), Klemetsen *et al.* (2003)
	S. trutta	Common	Cucherouset *et al.* (2003), Klemetsen *et al.* (2003)
Use of estuarine and marine environment for adult feeding migrations	*S. salar*	Common	Large-scale feeding migrations: Hanson & Quinn (1998), Klemetsen *et al.* (2003), Mackenzie *et al.* (2011)
	S. trutta	Common	Estuarine and coastal movements, large-scale migrations are rare: Euzenat (1999), Klemetsen *et al.* (2003)
Return to freshwater as immature fish without spawning	*S. salar*	Unknown?	
	S. trutta	Common	Elliott (1994), Klemetsen *et al.* (2003)

Table 4.1. Continued

Observed Life history variation	Species	Frequency of Occurrence	References
B) Reproduction			
Variable timing of spawning migration in adults during the year	*S. salar*	Common	Early ("spring") versus late-run adults; Klemetsen *et al.* (2003), Webb & McLay (1996)
	S. trutta	Common	Klemetsen et al. (2003), Davidson et al. (2006)
Variable age at first maturity	*S. salar*	Common	1 year in freshwater, after 1-5 years at sea: Klemetsen *et al.* (2003)
	S. trutta	Common	1-10 years in freshwater, < 1 year to 3 years at sea: Klemetsen *et al.* (2003)
Variable size at first maturity			
• "precocious" freshwater maturation in male parr	*S. salar*	Common	*ca.* 10 cm: Klemetsen *et al.* (2003)
	S. trutta	Common	*ca.* 10 cm: Klemetsen *et al.* (2003)
• "precocious" freshwater maturation in female parr	*S. salar*	Rare	*ca.* 10 cm: Prouzet (1981), Gibson *et al.* (1996)
	S. trutta	Rare	8-23 cm; Berg & Gausen (1988), Gibson *et al.* (1996)
• Variable adult size at first maturity	*S. salar*	Common	
	S. trutta	Common	Klemetsen *et al.* (2003)
			Klemetsen *et al.* (2003)
Repeat spawning activity	*S. salar*	Common	
	S. trutta	Common	Maximum Spawning is 5 times; Klemetsen *et al.* (2003)
			Maximum Spawning is 8 times; Harris (2002)
C) Feeding			
Trophic polymorphism with dietary specialisation	*S. salar*	Unknown?	
	S. trutta	Uncommon	Sonaghan, Gilleroo and Ferox trout: McVeigh *et al.* (1995)

Salmo trutta is native to Europe, North Africa and western Asia (Klemetsen *et al.*, 2003) and its natural distribution ranges from Iceland, northern Scandinavia and Russia in the North, with its western and southern limits defined by the coastal boundaries of Europe by the coast line of the Mediterranean Sea respectively and its eastern limits probably defined naturally by the Ural mountains and the Aral Sea (Elliott, 1994; Klemetsen *et al.*, 2003). However, *Salmo trutta* has also been introduced to over 24 countries outside its natural range Europe enabling them to become a successful global invader (Elliott, 1994; Westley and Fleming, 2011). For example, *Salmo trutta* have been introduced into Asia (*e.g.* Tibet, Hao and Chen, 2009: Pakistan, Khan *et al.*, 2011), South Africa (Cambray, 2003), North America (MacCrimmon and Marshall, 1968), South America (*e.g.* Falkands, McDowall *et al.*, 2001; Patagonia, Valiente *et al.*, 2010), Australasia (Townsend, 1996; Jackson *et al.*, 2004) and the sub-Antarctic region of the Southern Indian Ocean (Kerguelen Islands, LeComte *et al.*, 2013). The introduction of this species (whether purposely or accidently) into countries outside its usual native distribution has given it the dubious title of one of the "100 worst invasive alien species" by the IUCN Invasive Species Specialist Group (Lowe *et al.*, 2000; Westley and Fleming, 2011).

Both *Salmo trutta* and Atlantic salmon are well known for undertaking movements within both freshwater and between freshwater and the marine environment and for the homing ability of adult Atlantic salmon and sea trout to return to their natal rivers to spawn. At first feeding, fry of both species disperse away from the redd to establish feeding territories with smaller, potentially less-competitive fry dispersing further away to avoid competing with larger, more dominant fry (Daufresne *et al.*, 2005; Einum *et al.*, 2011; Einum *et al.*, 2011). At the parr stage, *Salmo trutta* appears to undertake more extensive movements within freshwater compared to Atlantic salmon and may move between tributaries, the river main stem and lakes depending on the geography of the catchment (Cucherousset *et al.*, 2003; Klemetsen *et al.*, 2003). After a variable period of time in freshwater (dependent on environmental constraints such as temperature and productivity on growth opportunity), most juvenile (*i.e.* excluding early maturing individuals; Table 4.1.) Atlantic salmon and the migratory component of the juvenile *Salmo trutta* population (*i.e.* sea trout) will undergo smoltification and migrate to sea as smolts (Stefansson *et al.*, 2008). However, many *Salmo trutta* do not go to sea and may undertake substantial movements as sub-adult and adult resident brown trout within

freshwater catchments (Cucherouset *et al.*, 2003; Klemetsen *et al.*, 2003) or may migrate into estuaries to feed for periods of time without fully migrating to sea (*i.e.* slob trout; Elliott 1989; Etheridge *et al.* 2008).

The movement patterns of post-smolts and adult salmon and sea trout within the marine environment, are less well known due to the difficulties of tracking fish at sea, however, Atlantic salmon have been the focus of much recent research effort (*e.g.* Mackenzie *et al.*, 2011; Hansen *et al.*, 2012; LaCroix, 2012; Sheehan *et al.*, 2012; LaCroix, 2013). On entering the sea, Atlantic salmon quickly move offshore (Jonsson and Jonsson, 2011*b*) and undertake large-scale migrations to feeding grounds in the Labrador Sea (west of Greenland), around the Faeroe Islands and in the Norwegian and Barents Sea (Reddin *et al.*, 2012). There is some discussion as to whether Atlantic salmon from all salmon rivers mix at sea and undertake large transoceanic migrations using the surface water currents of the North Atlantic Subpolar Gyre, the so called "Merry-go-round" hypothesis (Dadswell *et al.*, 2010), or whether fish from European salmon rivers north of 62°N migrate to the Arctic feeding grounds to the north of Europe (Faeroes, Norwegian and Barents Seas) whilst salmon from European salmon rivers below 62°N, together with North American salmon, migrate west to feed in the Labrador Sea (Holst *et al.*, 1999; MacKenzie *et al.*, 2012). Most evidence would support the latter hypothesis (reviewed in MacKenzie *et al.*, 2012).

In contrast to our knowledge of the movement patterns of Atlantic salmon, less is known about the marine movement patterns of post-smolt and adult sea trout as they have not been subject to the same degree of research effort. However, the available evidence suggests that their movement patterns are more complex. It is thought that they do not undertake large-scale, trans-oceanic feeding migrations like Atlantic salmon but remain within coastal waters or re-enter estuaries to feed (Pemberton, 1976; Elliott, 1994; Knutsen *et al.*, 2001; Klemetsen *et al.*, 2003; Knutsen *et al.*, 2004; Jonsson and Jonsson, 2011*a*; Jensen and Rikardsen, 2012). Sea trout may also re-enter freshwater (but not necessarily their natal river: Elliott, 1994; Klemetsen *et al.*, 2003) as immature subadults (variously known as finnock, whitling, herling or school peal) with returning mature adult sea trout without engaging in spawning (Elliott, 1994) and in some northerly populations in Norway sea trout may overwinter in freshwater (*e.g.* Jonsson, 1985; Berg and Berg, 1987).

Much of our understanding of the movement patterns of *S. salar* and *S. trutta* has been gained through studies using applied tags (see Chapter 1, section 1.2.) to track the movement of fish. Within freshwater, passive integrated transponder (PIT) tags and acoustic telemetry have been used to monitor the within-stream movements of parr, smolts and adult fish (*e.g.* Hawkins and Smith, 1986; Lucas *et al.*, 1999; Aarestrup *et al.*, 2003; Cucherousset *et al.*, 2003; Bendell *et al.*, 2005; Enders *et al.*, 2007; O'Donnell *et al.*, 2010; Debowski et al., 2011; Roy *et al.*, 2013) and to monitor the activity of fish on spawning grounds (Armstrong *et al.*, 2001). More recently, the use of strontium isotope ratios (^{87}Sr:^{86}Sr) has been used to track the movement patterns of fishes between different geologies within large river networks (Barnett-Johnson *et al.*, 2008; Walther *et al.*, 2011; Muhlfield *et al.*, 2012) but this approach has not been used to examine movement patterns in smaller (*i.e.* UK-size) catchments.

Movements of Atlantic salmon and sea trout post-smolts and adults in estuarine and coastal marine waters have been tracked using acoustic telemetry whilst larger-scale movements patterns have been traced using tag / recapture studies (reviewed in Drenner *et al.*, 2012; also see Bendall *et al.*, 2012; Ostergren *et al.*, 2012; Reddin *et al.*, 2012; Halfyard *et al.*, 2013; Lefevre *et al.*, 2013) and more recently by archival tags (Jensen and Rikardsen, 2012) and satellite tracking (Chittenden *et al.*, 2013). In addition, scientific studies tracking the marine movement patterns of sea trout have been supported by information supplied by recreational anglers from fish caught from the shore and in estuaries together with data / samples obtained from scientific research cruises, bycatch from commercial trawlers and from directed coastal and estuarine fisheries (*i.e.* commercial nets and traps) (ICES, 2010, 2011). Natural tags (see Chapter 1, section 1.3.) have also been used to look at movement patterns of Atlantic salmon at sea. For example, the transoceanic feeding migrations of European and North American adult Atlantic salmon have been tracked using bioaccumulated radiocaesium (Spares *et al.*, 2007), scale morphometry (Reddin and Friedland, 1999) and genetic analysis (Gauthier-Ouellet *et al.*, 2009). Most recently, stable isotopic composition of scales has been used to identify the marine feeding grounds analysis of Atlantic salmon (Mackenzie *et al.*, 2011) and to show that adult salmon from different rivers within the UK feed in different oceanic areas and even different phenotypes from the same stock (*i.e.* grilse *vs.* multi-seawinter salmon) have geographically-separate feeding areas (Mackenzie *et al.*, 2012). The stable isotopic composition of scales and otoliths have also been used in other marine fishes to examine

movement patterns (*e.g.* Rooker *et al.*, 2008) and thus this technique may be a promising tool for tracking marine migrations. However, to date, this technique has been used in large-scale, trans-oceanic migrations (*e.g.* Rooker *et al.*, 2008; Mackenzie *et al.*, 2012) and has not been tested on a smaller geographical scale, for example to try to track the movement patterns of fishes in continental shelf waters.

In recent years there has been a dramatic increase in the use of trace element microchemistry to describe differences in the chemical composition of otoliths from fish inhabiting chemically-distinct bodies of water, for example between different rivers, estuaries, or coastal nursery areas to identify the natal origin of fishes (see Chapter 1, section 1.4.). Although there are many studies showing that geographically-isolated populations of fish differ in their otolith chemistry and that these differences can be used to identify individual fish back to their source population using discriminant function analysis (*e.g.* Veinott and Porter, 2005, 2013; Walther and Thorrold, 2008; Olley *et al.*, 2011; Ramsay *et al.*, 2011), there are very few studies which have collected fish of unknown provenance from the wild and tried to assign them back to a putative source population based on an established baseline of source populations (Walther and Thorrold, 2010; Olley *et al.*, 2011; Veinott *et al.*, 2012; Chittaro and Hogan, 2013; Chittaro *et al.*, 2013). The Irish Sea is one of the coastal shelf seas (or Regional Seas) found in the United Kingdom with a surface area of 58,000 km^2 (Vincent *et al.*, 2004). The movement of water from the Atlantic Ocean into and out of the Irish Sea can occur through St George's Channel in the south and the North Channel in the Northwest Irish Sea (Vincent *et al.*, 2004) and therefore the Irish Sea presents a semi-enclosed shelf sea within which the movement patterns of fishes at sea and their geographical origins (*e.g.* coastal water nursery areas or freshwater origins for anadromous fish) could potentially be studied. Sea trout are not thought to undertake long-distance oceanic feeding migrations (see Figure 4.2.) like Atlantic salmon: the evidence from tracking studies suggests that they remain within coastal waters or re-enter estuaries to feed (Pemberton, 1976; Elliott, 1994; Knutsen *et al.*, 2001; Klemetsen *et al.*, 2003; Knutsen *et al.*, 2004; Jonsson and Jonsson, 2011*a*; Jensen and Rikardsen, 2012). Therefore, it may be possible to track the movement patterns of sea trout within the Irish Sea using chemical tags to determine the extent of their migrations away from their natal rivers.

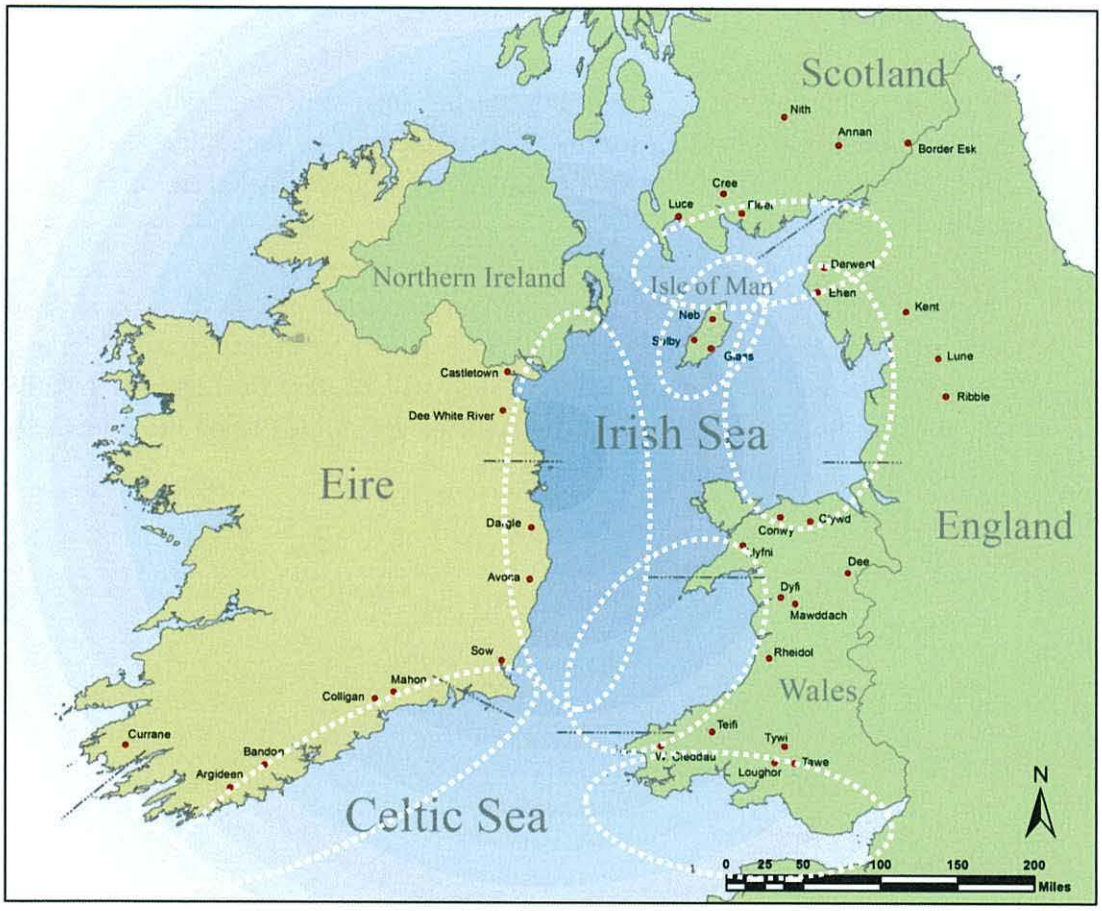


Figure 4.2. Map of the Irish Sea showing the hypothetical feedings areas (white dashed ovals) for sea trout captured within the marine zones for the present study, based on the hypothesis that sea trout will remain in coastal waters close to their natal region to feed.

4.1.1. Aims

The aims of this study are outlined as follows:

1. To describe the differences in the trace elemental concentrations within the otoliths of *Salmo trutta* L. parr collected from 36 rivers located within 9 regions (see Figure 4.3.) in the Irish and Celtic Sea region and to determine whether individual fish can be assigned back to their river or region of origin using otolith microchemistry.

2. To test the robustness of the freshwater baseline, parr collected from the 36 rivers but not used to establish the freshwater baseline will be randomly selected and allocated "blind" (*i.e.* with no prior knowledge of the actual river of origin) to putative river of origin.

3. The trace element microchemistry of the parr and adult phases of the otoliths of adult sea trout caught in coastal waters in the Irish Sea (East coast of Ireland, Solway Firth, Isle of Man and North Wales) will be described and the putative region of freshwater origin of each fish will be determined using the established parr freshwater baseline.

4.2. Methods

4.2.1. Juvenile trout parr specimens

Juvenile brown trout parr were collected as part of the Celtic Sea Trout Project (CSTP) during the summer of 2010 from 33 of the main sea trout producing rivers in SW Scotland, NW England, Wales, Isle of Man and the east coast of Ireland which flow into the Irish and Celtic Seas. In addition a further 3 rivers (Cree, Argideen and the Bandon) sampled during the summer 2011 were also included giving a combined total of 36 rivers in 10 sub-regions around the Irish / Celtic Seas (see Table 4.2. and Figure 4.3. for GPS geographical locations). Samples were collected using gill nets, fyke traps or electro fishing methods by collaborating CSTP partners. Trout parr were euthanized using approved techniques for use in the Republic of Ireland of the UK. In the UK Home Office Schedule 1 methods were used, *i.e.* either sharp blow to the head or overdose in a strong anaesthetic solution (*e.g.* 2-phenoxyethanol, clove oil or benzocaine) followed by transaction of the spinal cord and destruction of the brain. Biological measurements were taken for each juvenile trout parr: total length (TL, ± 0.1 cm) and body wet weight (TW, ± 0.1 g). Parr were then stored frozen at -20°C until transported (on ice) to the School of Ocean Sciences where they were stored at -30°C until processed. Although not aged, the size ranges of fish sampled (Table 4.2.) suggest that 1 and 2 year old trout parr were sampled.

4.2.2. Adult sea trout specimens

Adult sea trout were captured in the coastal waters of the Irish and Celtic Seas during 2010 – 2012 using a variety of fishing techniques; including surface water pelagic trawling (using 2 CSTP-hired commercial fishing vessels), recreational fishing (angler rod and line) and four netting techniques (*i.e.* seine, gill, stake and haaf netting). As part of the CSTP programme, the coastal waters of the Irish and Celtic Seas were divided up into 18 coastal marine zones (MZs, see Figure 4.3.) in order to provide a stratified sampling approach for the project to collect samples from throughout the region for life-history, genetic and microchemistry analysis. For the purposes of this microchemistry study, a total of 240 adult sea trout caught from five marine zones located in S-W

Scotland (MZ10), N Wales (MZ13 and MZ14), Ireland (MZ6) and the Isle of Man (MZ23) were selected for analysis (see Figure 4.3., Table 4.3.). These regions were selected, in part, because these were the regions where most sea trout were caught, but also because they gave a good geographic spread of coastal regions within the Irish Sea.

Adult trout were euthanized on capture using the approved techniques in Republic of Ireland or UK (see section 4.2.1.), with commercially-caught trout supplied as dead samples. Total length to the nearest millimeter (TL, ± 0.1 cm) and total weight to the nearest gram (TW, ± 1 g) were measured in the field where practicable, with fish whose biological measurements were not taken at capture measured during the processing stage. Fish were stored frozen at -20°C where practicable after death and transported on ice to the School of Ocean Sciences where they were stored at -30°C until required for dissection and otolith extraction.

Table 4.3. Summary for the marine-caught *Salmo trutta* for the 5 marine zones used in the adult microchemistry analysis. Data are provided on the number of fish collected in each marine zone between 2010 and 2012. For locations of each marine zone, see Figure 4.3.

Marine Zone N° (MZ)	Location	Regional Zone	N° of Adults	Years Collected
MZ-06	East coast of Ireland	South Skerries	67	2010 = 2 2011 = 39 2012 = 27
MZ-10	South West Scotland	Solway Firth	72	2010 = 45 2011 = 27
MZ-13	North Wales	North Wales	24	2010 = 4 2011 = 20
MZ-14	North Wales	North Wales	32	2010 = 20 2011 = 12
MZ-23	Isle of Man	Isle of Man	36	2010 = 13 2011 = 23

Table 4.2. Details of river site locations (River, site name and GPS coordinates for middle of stream sampled) where juvenile *Salmo trutta* parr were collected for the Irish Sea microchemistry baseline. Twenty five fish were sampled at each site (unless otherwise indicated). Size data are presented as mean total length (TL) ± sd plus the minimum-maximum size range. Rivers divided into 10 sub-regions are indicated by shading.

Region and River number	River	Site name	GPS Location	Year sampled	TL range (mm)	Mean TL (mm)
SW Scotland						
1	Luce	Lady Burn	54.878, -4.810	2010	84 - 109	91.5 ± 5.4
2	Cree*	Penkiln Burn	55.019, -4.425	2011	85 - 170	126.1 ± 19.7
3	Fleet	Barley Burn	54.895, -4.216	2010	80 - 109	89.1 ± 6.8
4	Nith	Wanlock Water	55.414, -3.817	2010	90 - 128	114.2 ± 10.9
5	Annan	Windyhill Burn	55.212, -3.617	2010	75 - 135	95.6 ± 11.5
6	Border Esk	Meggat Water	55.228, -3.083	2010	100 - 156	119.8 ± 13.2
NW England						
7	Ehen	Kirk Beck	54.465, -3.498	2010	94 - 130	109.8 ± 9.2
8	Derwent	Marron	54.573, -3.448	2010	102 - 148	124.7 ±15.9
9	Kent	Lambrigg Beck	54.361, -2.668	2010	103 - 153	130.4 ± 14.2
10	Lune	Ellergill Beck	54.442, -2.556	2010	75 - 135	125.6 ± 16.6
11	Ribble	Twiston Beck	53.897, -2.299	2010	100 - 156	118.4 ± 13.2
Wales						
12	Dee	Eglwyseg	52.985, -3.185	2010	104 - 176	122.7 ± 16.6
13	Clwyd	Deunant	53.192. -3.562	2010	95 - 143	116.1 ± 12.4
14	Conwy	Roe	53.214, -3.848	2010	100 - 143	122.3 ± 11.2
15	Llyfni	Nant Tal-y-Mignedd	53.055, -4.201	2010	73 - 128	91.2 ± 15.0
16	Mawddach	Nant Pwll y Gele	52.762, -3.841	2010	76 - 116	90.6 ± 11.1
17	Dyfi	Cerist	52.726, -3.705	2010	81 - 120	99.6 ± 11.1
18	Rheidol	Melindwr	52.402, -3.974	2010	102 - 161	137.7 ± 14.7
19	Teifi	Nant Bargod	52.026, -4.399	2010	111 - 157	129.8 ± 11.8

Table 4.2. Continued.

Region and River number	River	Site	GPS Location	Year	TL range (mm)	Mean TL (mm)
Wales						
20	W. Cleddau	Anghof	51.917, -4.935	2010	111 - 159	138.7 ± 13.8
21	Tywi	Sawdde	51.898, -3.805	2010	100 - 158	121.5 ± 15.9
22	Loughor	Aman	51.804, -3.898	2010	117 - 159	144.7 ± 11.0
23	Tawe	Main river	51.801, -3.707	2010	104 - 170	129.3 ± 16.3
Ireland						
24	Dee (White River)**	Main river	53.843, -6.395	2010	132 - 167	149.9 ± 9.7
25	Castletown	Main river	54.031, -6.445	2010	112 - 169	149.5 ± 12.0
26	Dargle	Main river	53.155, -6.196	2010	80 - 116	93.9 ± 9.3
27	Avoca	Derry	n/a	2010	103 - 144	122.1 ± 11.5
28	Sow**	Main river	52.396, -6.472	2010	121 - 153	138.5 ± 8.7
29	Mahon	Main river	52.212, -7.485	2010	103 - 144	122.1 ± 11.5
30	Colligan	Main river	52.171, -7.663	2010	n/a	n/a
31	Argideen	Main river	51.647, -9.022	2011	100 - 139	117.5 ± 11.6
32	Bandon	Brinney	51.783, -8.702	2010	99 - 152	123.9 ± 12.5
33	Currane	Finglas	51.804, -10.141	2010	90 - 165	114.2 ± 19.1
Isle of Man						
34	Sulby	Main river	54.316, -4.486	2010	84 - 137	103.6 ± 20.0
35	Glass	Main river	54.204, -4.659	2010	88 - 122	96.9 ± 9.1
36	Neb	Main river	54.154, -4.502	2010	78 - 120	88.5 ± 9.1

* n = 29, ** n = 15, n/a = data not available.

Figure 4.3. Map indicating locations of the 36 rivers sampled around the Irish and Celtic Seas for the Celtic Sea Trout Project. Boundary markers (---) indicate sub-division of the locations into 10 regions. Designated marine zones (MZ) within the Irish Sea are defined by red boundary markers, with MZ 06, 10, 13, 14 and 23 used in this study.

4.2.3. Equipment preparation

Before samples were run at the National Oceanographic Centre, Southampton (NOCS) and prior to otolith digestion, acid-washing protocols implemented at NOCS (Dr. Cooper *Pers comm.*, 2010) were observed for all equipment used in the preparation and digestion of the left sagittal otolith, with all preparation and digestions performed in a class 100 clean room. Scintillation bottles were acid-washed in 10% (1.7 M) HNO_3 for 2 days (turning over after 24 hours to wash the upper section of the bottle and cap), emptied, triple-rinsed in ultra-pure Milli Q water (Millipore™ hereafter referred to as Milli Q) and dried for 24 hours in a positive flow cabinet in a class 100 clean room. All pipette tips and disposable 0.5ml pipettes were triple acid-washed in 20.7% (6 M) HCl acid and finally triple-rinsed in Milli Q before use.

4.2.4. Extraction of otoliths

The methods used to extract the otoliths from the trout in this study were the same as those outlined in section 2.2.6., with the following modification. Juvenile trout parr samples were removed from the freezer (in batches of 10) and allowed to thaw for approximately 10 minutes, whilst adult sea trout samples were removed from the freezer (in batches of 5) and allowed to thaw for approximately 30 minutes.

4.2.5. Otolith dilution (Parts I and II)

The methods used to dilute the trout otoliths in this study in the two stage dilution process were the same as those outlined in section 2.2.7. and 2.2.7.1. with the following modifications. (1) the left sagittal otoliths were used in all analysis using sb-ICP-MS. (2) during the first dilution stage, 0.5 ml of 17.5% (3 M) HNO_3 was initially pippeted and left to allow time for the otolith to effervesce and dissolve. (3) to assist in the calibration of the machine and the samples, the digestion acid was spiked with a known concentration of 3 elements (10ppb Indium (In), 10ppb Rhenium (Re), 20ppb Beryllium (Be) according to NOCS protocols (Dr. Cooper *Pers comm.*, 2010). (4) before the otolith-acid solution could be transferred from the 1.5 ml micro-centrifuge tubes to individually labeled pre acid-washed scintillation vials, the micro-centrifuge tubes containing the otolith-acid

solution were spun in a centrifuge for approximately 40 seconds (spin rate 6,600 rpm, force 2,200g) to allow the otolith-acid solution to mix (Dr. Cooper *Pers comm.*, 2010). The otolith-acid solution was then pipetted into the scintillation vials using acid-washed 0.5 ml disposable pipettes. To ensure any remaining residue within the micro-centrifuge tubes and the disposable pipettes was not lost, tubes and pipettes were rinsed with 1 ml of 2% (0.3 M) HNO_3 (spiked with 10ppb In, 10ppb Re, 20ppb Be), with the micro-centrifuge tubes (containing the 1 ml of 2% HNO_3 acid), mixed for a further 40 seconds before the remaining residue was pipetted into the scintillation vial containing the otolith-acid solution. (5) The otolith-acid solution was diluted one final time by pipetting 5.0 ml of 2% (0.3 M) HNO_3 acid (spiked with In, Re, Be) to reduce the otolith-acid solution concentration to the required 50 ppm Ca before being run through the ICP-MS.

4.2.6. Otolith preparation for analysis using laser ablation inductively-coupled plasma mass spectrometry

The left sagittal otoliths from the adult sea trout were used in all analyses using LA-ICPMS. Left adult sagittal otoliths were embedded in Kleer-Set™ polyester resin (MetPrep) using a polyethylene mould (10mm depth 8mm Ø) and manually ground to the plane of the primordia (sulcal side) using 1200 to 2500 grade silicon carbide wet / dry abrasive paper. Left sagittal otoliths were individually mounted on acid-washed glass slides (which fit the dimensions of the laser ablation cell, Dr. Chenery *Pers Comm.*, 2012) using Crystalbond™, triple-rinsed in Milli Q (to remove any remaining residue from the grinding process), dried in a laminar positive flow cabinet and stored in individually-labelled plastic envelopes prior to LA-ICPMS.

4.3. Sample measurement and data processing

4.3.1. Juvenile brown trout: sb-ICP-MS measurement and data processing

The methods used to measure and screen the data produced from the juvenile brown trout otoliths using solution-based ICP-MS are outlined in detail in section 2.3.1. with the following modifications:

(1) 17 elements were measured using sb-ICP-MS, The assessment of which elements to measure in the brown trout parr otoliths was decided in discussion with Dr. C. Trueman (Co-supervisor NOCS for CSTP) and Dr. M. Cooper after reviewing published data on microchemistry of fish otoliths. In total, 17 elements were targeted for analysis using ICPMS: Li, Mg, Ca ^{43}, Cr ^{52}, Mn, Co ^{59}, Ni ^{60}, Cu, Zn, Rb, Sr, Cd ^{111}, Ba, Ce ^{140}, Gd ^{157}, Pb ^{208}, U ^{238}. The selected elements were chosen because (a) the ease with which they could be assayed and (b) the sensitivity of the mass spectrometer (with regards to the limits of detectability) to determine the strength (*i.e.* concentration) and quality (*i.e.* limits of detection) of those particular elements which may be contained within the juvenile brown trout parr otoliths.

(2) All measurements (*i.e.* otolith samples, internal standards, quality control standards and system blanks) were run on a Thermo Scientific X-Series II inductively-coupled plasma mass spectrometer equipped with an ICP-MS auto sampler. All samples (including standards) being assayed were injected into the ICP-MS and were aerosolized in a gas plasma produced in a quartz torch at 5000° Kelvin, with the sample ions drawn from the plasma into an off-axis high-performance quadrupole mass analyser. All data were assessed for the effects of possible polyatomic ionization interference. Furthermore to reduce this occurrence, the Thermo X-Series II machine uses a 3rd generation collision-reaction cell incorporating kinetic energy discrimination into the spectrometer (Dr. Cooper *Pers comm.*, 2011).

The raw data produced from the sb-ICP-MS analysis were examined using the Thermo Scientific X-Series II integrated software. One of the 17 elements analysed (Ni) was subsequently removed (see Table. 4.4.) due to possible background ion effects (sample interface cones are made of nickel; Dr. Cooper *Pers comm.*, 2011). In addition two elements (Ce and Gd) were also omitted from the final data set due to their extreme rarity within the natural environment ("Rare Earth Elements") but were run as a test of the precision of the ICP-MS (Dr. Cooper *Pers comm.*, 2011).

Table 4.4. Elements assayed (in descending order of their relative atomic mass) in the juvenile brown trout parr otoliths. Background elements and elements observed at their limits of detection (LOD) using multi-element solution standard and internal standards for ICP-MS are indicated (*background ion effect, # elements exhibiting LOD).

Elements				
$Li^{\#}$	Mn	Zn	Ba	$^{238}U^{\#}$
Mg	$Co^{\#}$	Rb	**$Ce^{\#}$**	
^{43}Ca	Ni^{*}	Sr	**$Gd^{\#}$**	
Cr	Cu	$^{111}Cd^{\#}$	^{208}Pb	

Elements in bold are rare Earth elements and would probably not be observed above the LOD within the samples (Dr. Cooper *Pers comm.*, 2011).

4.3.2. Adult sea trout: LA-ICP-MS measurement and data processing

The methods used to screen the data produced from the adult sea trout otoliths are outlined in detail in section 2.3.1. with the following modification: 17 elements - Li, B, Na, Mg, Al, K, Ca, Cr, Mn, Fe, Cu, Zn, Rb, Sr, Sn, Ba, Pb - were measured for analysis using laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS) (see Table 4.5.).

Table 4.5. Elements targeted (in order of their relative mass) using laser ablation (LA) ICP-MS on adult sea trout otoliths. Elements observed at their limits of detection (LOD) using international standard NIST 612 (National Institute of Standards and Technology, USA) with NIST 610 used as a secondary reference material are indicated (#elements exhibiting their LOD).

Elements				
$Li^{\#}$	$Al^{\#}$	Mn	$Rb^{\#}$	$^{208}Pb^{\#}$
$B^{\#}$	K	$Fe^{\#}$	Sr	
Na	^{43}Ca	$Cu^{\#}$	$Sn^{\#}$	
Mg	$Cr^{\#}$	Zn	Ba	

Sagittal otoliths were laser ablated (sulcus facing upwards) to determine their trace elemental concentrations using a Class 4 Nd:YAG solid state 193 nm excimer lamp-pumped laser ablation system (New Wave Research, U.S.A) attached to an *in situ* Agilent 7500c inductively-coupled plasma mass spectrometer (ICP-MS) at the British Geological Survey, Keyworth. Each slide held between 24-32 otoliths which were analyzed in batches of 8, with each slide containing approximately equal numbers of adult otoliths

from each of the marine zones to be analysed arranged in random order on the slide (Ramsay *et al.*, 2011). This method of analysis removed the possibility of systematic error which could arise as a result of a variation between runs (see Brophy *et al.*, 2003). Mounted otoliths were housed in an airtight ablation cell clamped to an adjustable motorized stage of a binocular microscope and CCD video camera coupled to a computer monitor. Otolith images were illuminated using a 3-way light source (either transmitted, ring or coaxial) to allow the precise focus and identification of the sample area to be ablated with the observations of the analysis viewed on the monitor.

Adult sea trout otoliths were ablated across the surface of the sulcus side of the otolith from the primordium (*i.e.* area surrounded by the nucleus, as defined by Campana and Nielson, 1985) to the dorsal edge at a rate of 4 µm s^{-1} using a 50 µm Ø laser spot size firing at a repeat rate of 10Hz with an irradiance of 0.87 GW/cm^2 and a fluence of 3.53 J/cm^2 with a 30 second wash out between samples. Ablated material from the otolith was carried to the ICP-MS in a flow of helium (0.80 l/min^{-1}) where it was combined with a stream of argon carrier gas (0.85 l/min^{-1}) before reaching the ICPMS.

To correct for changes in ablation efficiency between scans and along whole traverses, and to assess instrument sensitivity (possible machine drift *e.g.* non-spectral interferences resulting in signal suppression / enhancement), element:Ca ratios were compared to a standard reference material. Calibration was conducted using the international standard NIST 612 (National Institute of Standards and Technology, USA) with NIST 610 used as a secondary reference material. Raw counts (cps) of the 17 elements from each of the ablated otoliths were transferred using the Agilent Proprietary ICP-MS software (Agilent Technologies, U.S.A.) and processed off line using the Iolite software package extension in Igor Pro (Igor Pro 6.2: WaveMetrics).

4.3.3. Analytical figures of merit including limits of detection

The methods used to determine the limits of detection for the elements measured in this study are outlined in section 2.3.2., with the following modifications:

(1) following the assessment of LOD for each of the 17 elements measured in the brown trout parr otoliths, the background element Ni and 6 elements falling below LOD - Li, Co, Cd, Ce, Gd and U - were omitted from any further analysis.

(2) of the 10 remaining elements above LOD in the trout parr otoliths - Mg, Ca, Cr, Mn, Cu, Zn, Rb, Sr, Ba and Pb - four elements (Cr, Cu, Zn and Pb) were found to vary in their concentrations when compared to internal standards and standards used to calibrate for possible machine drift (Dr. Cooper *Pers comm.*, 2011) and were subsequently removed from the final statistical analysis.

(3) of the 17 elements (see Table 4.5.) measured in the material ablated from adult sea trout otoliths, 9 - Na, Mg, K, Ca, Mn, Zn, Sr, Sn, Ba - indicated concentrations above the LOD with the 8 elements falling below LOD (Li, B, Al, Cr, Fe, Cu, Rb, Pb) omitted from any further analysis.

4.3.4. Elemental outliers & corrections

The methods used in the identification and correction of outliers in this study followed those outlined in section 2.3.3. In total, six elements - Mg, Ca, Mn, Rb, Sr, and Ba - identified in juvenile trout parr otoliths and nine elements – Na, Mg, K, Ca, Mn, Zn, Sr, Sn, Ba - identified in adult sea trout otoliths were analysed, outliers observed in the original raw data identified and corrected for and assessed for their %RSD. Each element was then standardized to calcium to produce element:Ca ratios ($\mu g\ g^{-1}$).

4.4. Statistical analyses

4.4.1. Data transformations

The data transformation methods used in this study are outlined in detail in section 2.4.1., in summary:

Element concentrations measured from parr otolith samples were Log_{10} transformed prior to statistical analysis to meet assumptions of normality and homoscedasity. Despite Log_{10} transformation, the elements Mg, Mn, Rb, Sr and Ba failed normality (Anderson Darling test, $P > 0.05$) and homoscedasticity tests (Levene's test $P < 0.05$). However, due to number of fish measured in this study ($n = 665$) and the sensitivity of the AD test of normality to large sample sizes, graphical plots were used to assess normal distributions with only minor deviations indicated.

Elemental concentrations measured from adult trout otoliths were Log_{10} transformed prior to statistical analysis to meet assumptions of normality and homoscedasity, with Na, Mg, K and Ba meeting both assumptions. Despite Log_{10} transformation elements Mn, Zn and Sr failed normality (Anderson Darling test, $P > 0.05$), with Mn, Sr and Sn failing homoscedasticity tests (Levene's test $P < 0.05$). However, due to number of fish measured in this study (n = 231) and the sensitivity of the AD test of normality to large sample sizes, graphical plots were used to assess normal distributions with only minor deviations indicated for the elements Mn and Zn.

4.4.2. Assessment of juvenile trout data

The methods of statistical analysis applied to the juvenile parr data are outlined in detail in section 2.4.2., with the following modifications / additions:

Statistical analyses were performed on the elements most commonly used in studies of this nature: Mg, Mn, Sr and Ba (see Wells *et al.*, 2000; Wells *et al.*, 2003; Ramsay *et al.*, 2011; Ramsay *et al.*, 2012). However, because rubidium (Rb) can be biologically mediated (*i.e.* found within animal tissue) and in some instances mimic potassium (K) in in its distribution and excretory patterns (Hays and Swenson, 1985; Soetan *et al.*, 2010), the element rubidium was removed from the final statistical analyses. Furthermore, Rb has been shown to be present as single-valent inclusions within the otolith and it has been suggested that it may have little or no biological relevance in microchemistry studies because of its extremely labile nature (Rooker *et al.*, 2001).

To analyse and quantify any variation observed between brown trout parr otolith trace elemental concentrations and multi-elemental fingerprints between rivers and between sub-regions (see Figure 4.2.1.), a multivariate MANOVA was performed. Examination of individual trace element concentrations of otoliths between rivers and sub-regional boundaries was conducted using an ANOVA on each of the four Log_{10} transformed elements - Mg, Mn, Sr and Ba. Following significant ANOVAs on the sub-regional trace element data, Bonferroni *post-hoc* pairwise comparisons were made between regions to identify where significant differences occurred. However, multiple *post-hoc* pairwise comparisons (Bonferroni test) were tested for rivers in the present study but not assessed due to the large number of comparisons required to be conducted between each of the 36 rivers and the large uninformative data set generated.

To assess the accuracy with which juvenile brown trout parr were classified back to their natal rivers / sub-regions based on their otolith chemical signatures, discriminant function analyses (DFA) were performed (Wells *et al.*, 2000; Clarke *et al.*, 2007; Ramsay *et al.*, 2011), using a quadratic discriminant function analysis (QDFA). Firstly, the use of QDFA does not require data to be homoscedastic (within-group covariance matrices are assumed unequal; Lachenbruch, 1975) and secondly it has been shown to be relatively robust to the assumptions of normality and homogeneity being violated (see Lachenbruch, 1975; Lachenbruch and Goldstein, 1979; Klecka, 1980, also see McGarigal *et al.*, 2000 for normality assumptions using QDFA).

QDFA involves the use of the discriminant functions to classify the same samples used to develop those functions as an "original" classification. Furthermore, a "cross-validation" classification analysis was run in conjunction with the original classification where one sample is left out of the dataset before establishing the original discriminant functions and then classifying the removed sample (see Sharma, 1996). QDFA can be run if minor deviations from normality are observed within the dataset (Leakey *et al.*, 2008; Ramsay *et al.*, 2011). However, due to the assumptions not being met because the data was found to be heteroscedastic a further analysis was run using Random Forest (RF), (R Foundation for Statistical Computing, Vienna, Austria RF: Breiman, 2001) to validate both the overall and group classification results observed using QDFA.

Random Forest is a classifier consisting of a collection of tree-structured classifiers using independent identically-distributed random vectors and each tree casts a unit vote for the most popular class at input (Breiman, 2001). Furthermore, it has been shown to perform extremely well with large unbalanced data sets without pre-processing (*e.g.* rescaled, transformed or modified) when compared to other classifiers such as discriminant function analysis (see Liaw and Wiener, 2002). Subsequently there is no need for cross-validation or separate tests set to get unbiased estimates of the generalized error (test error) (Liaw and Wiener, 2002). The uses of these two statistical methods were to address the above problems of non-normality and heteroscedasticity observed in the data. Furthermore, assessment of classification methods using alternative analyses has been shown to be robust with results using non-parametric classification tests validating the overall group classification achieved using QDFA. For example, Ramsay *et al.* (2011) used multinomial linear regression as a validation test assessing potential effects of non-normality using QDFA.

To evaluate the performance of the discriminant functions (in regards to their classification results), a Cohen's kappa analysis (Titus *et al.*, 1984) was performed. The use of this statistical method assesses the probability of the classifications being assigned purely by chance. Kappa's statistic values range between 0 and 1, with 0 indicating no improvement over the chance classification and 1 indicating perfect agreement with the classification results (Barnett-Johnson *et al.*, 2008; Ramsay *et al.*, 2011).

Principal component analysis (PCA) plots were used to provide visual representation of the classification observed for the QDFA results of individual fish back to their river and region of origin.

4.4.3. Assessment of adult trout data

The methods of statistical analysis applied to the adult sea trout are outlined in detail in section 4.4.2, with the following modifications / additions:

Parametric analysis using MANOVA was performed on the elements Na, Mg, K, Zn and Ba to quantify variations in the multi-elemental fingerprints observed between the five marine zones (Figure 4.3.). Following the MANOVA, individual examination of trace elemental concentrations were conducted using ANOVA followed by Bonferroni *post-hoc* pairwise comparisons between marine zones to identify where significant differences occurred.

Non-parametric analyses (Kruskal-Wallis and Mann-Whitney U tests) were conducted on the elements failing the assumptions for parametric analyses (*i.e.* Mn and Sn).

Assessment of the accuracy with which adult trout were classified back to their marine zones (using the marine growth phase, see Figure 4.4.) were performed using QDFA on the elements Na, Mg, K, Mn, Zn, Sn and Ba. Using the established freshwater parr base-line classification accuracy in assigning adult trout to their natal sub-region (using their freshwater growth phase, see Figure 4.4.) was performed using the elements which were included in the baseline analysis, *i.e.* Mg, Mn, Sr and Ba.

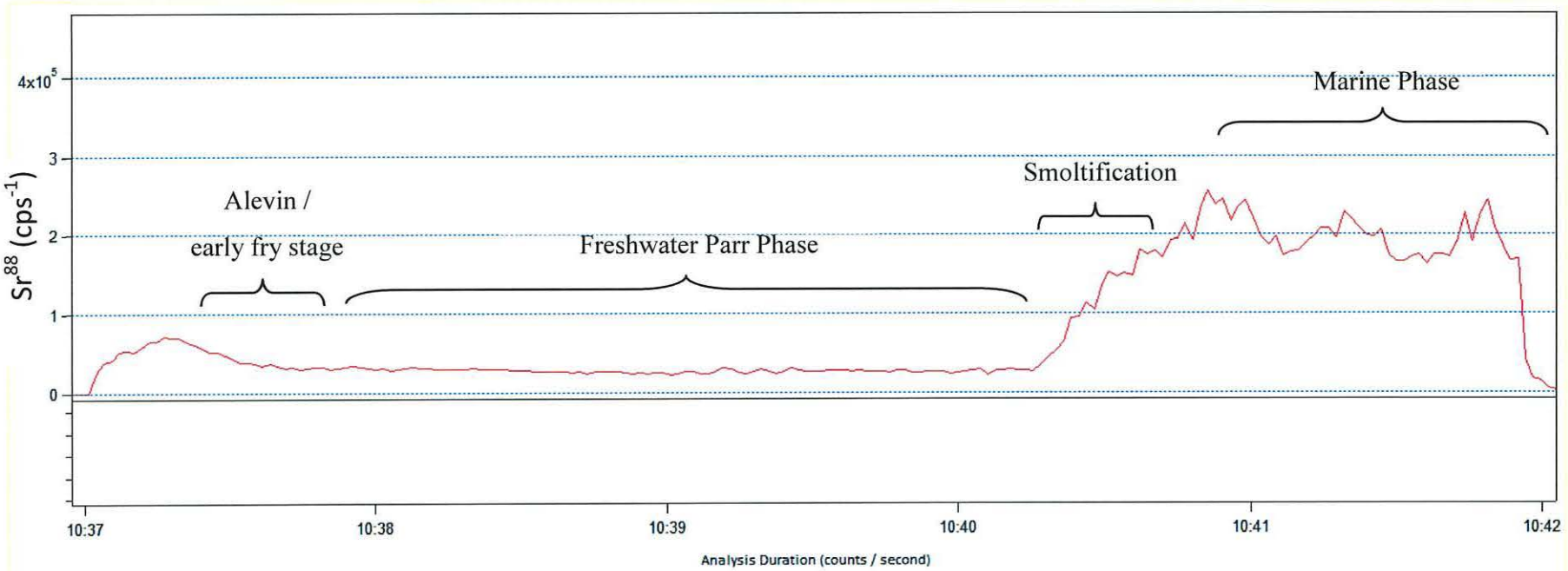


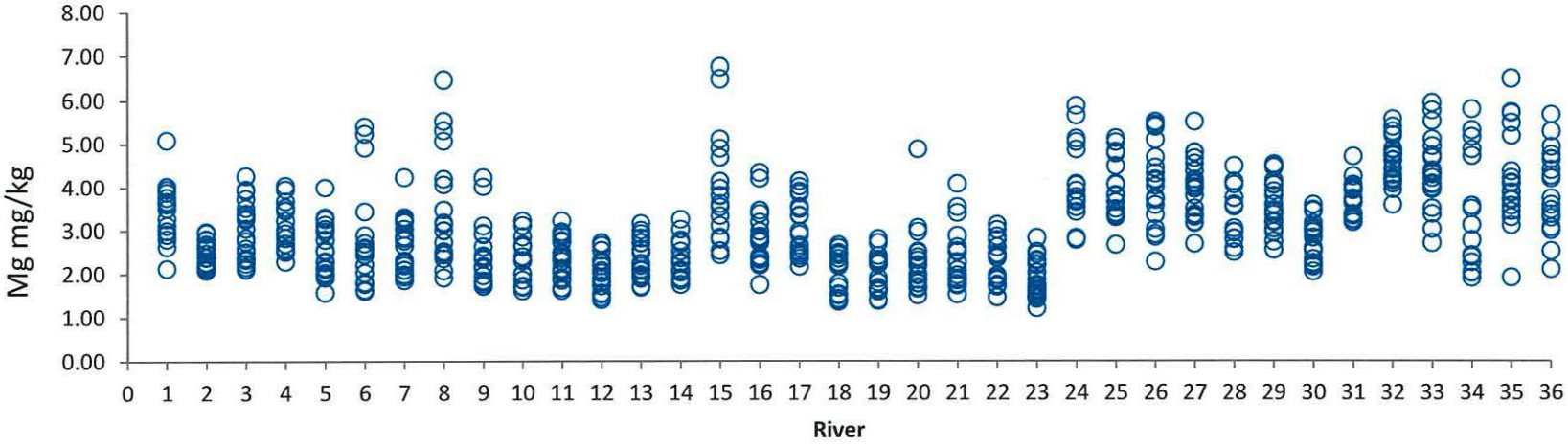
Figure 4.4. Relationship of strontium^{88} concentration (as counts s^{-1}) along a transect through the otolith of a marine-caught adult sea trout showing how it is possible to identify the different phases of the life cycle based on measured strontium values.

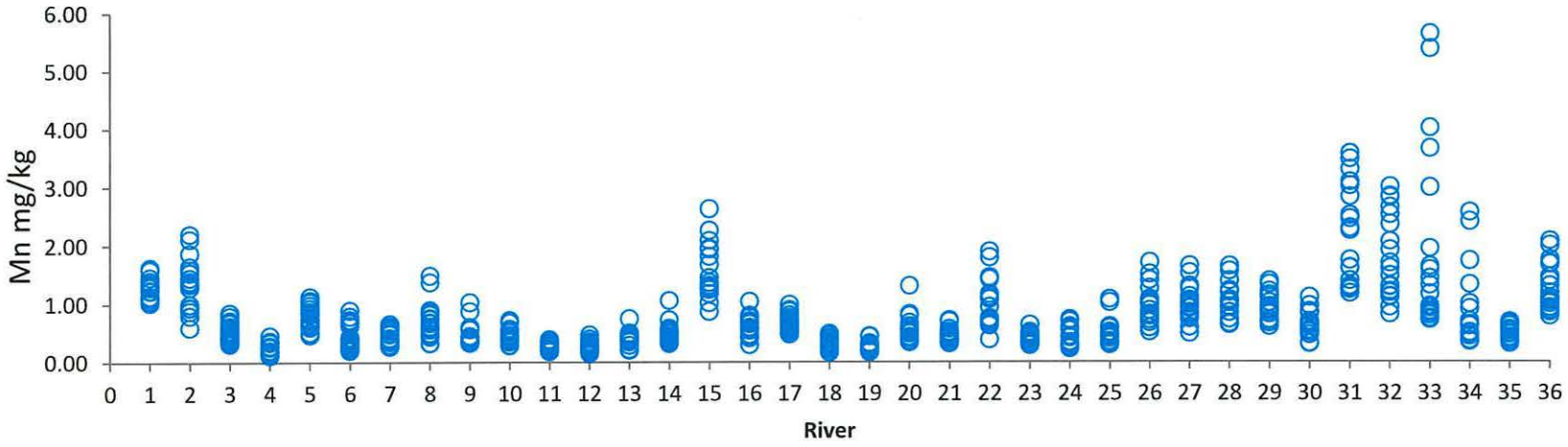
4.5. Results of parr analyses

4.5.1. Classification of trout parr to their river of origin

The mean (± sd) elemental concentrations (expressed as mg/kg) in the sagittal otoliths of juvenile trout parr for each of the four elements are presented in Figure 4.5. to provide a visual assessment of the elemental concentrations for each of the 36 rivers sampled. Differences in otolith chemistry between rivers can be seen with a 2.4 fold difference in Mg (1.900 - 4.606 mg/kg), a 10.6 fold difference in Sr (25.345 - 151.542 mg/kg) and order of magnitude differences in Mn (10.6 fold; 0.216 – 2.286 mg/kg) and Ba (14.3 fold; 0.174 – 2.493 mg/kg) respectively (see Figure 4.5. and Table 4.6). Geographical patterns in otolith chemistry could be observed between rivers, most notably for Mg between the rivers 1-23 (Scotland, England and Wales) and the rivers 24-36 (Eire and Isle of Man). Similarly, the element Mn and the rivers from the south west Eire and the Isle of Man (31-36) and the remaining rivers (1-30). One river, the Currane in south west Ireland (River 33 in Figure 4.5.) was notable for exhibiting high Mn, Sr and Ba concentrations compared to the other rivers. The location, *i.e.* sub-region, of river appeared to influence otolith microchemistry with rivers in SW Scotland, NW England and Wales having lower Mg and Mn concentrations than rivers in Ireland or the Isle of Man (Figure 4.6.).

Significant differences were observed for the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba in the sagittal otoliths of trout parr between each of the 36 rivers (MANOVA: using Willks' criterion: $F_{140, 2495} = 72.003$, $P < 0.001$). Individual ANOVA's conducted using each of the four elements Mg, Mn, Sr and Ba indicated highly significant differences in concentrations for each element between the 36 rivers (Mg: $F_{35, 664} = 27.15$, $P < 0.001$; Mn: $F_{35, 664} = 55.59$, $P < 0.001$; Sr: $F_{35, 664} = 194.11$, $P < 0.001$; Ba: $F_{35, 664} = 117.12$, $P < 0.001$).





(Figure 4.5. and caption are continued overleaf)

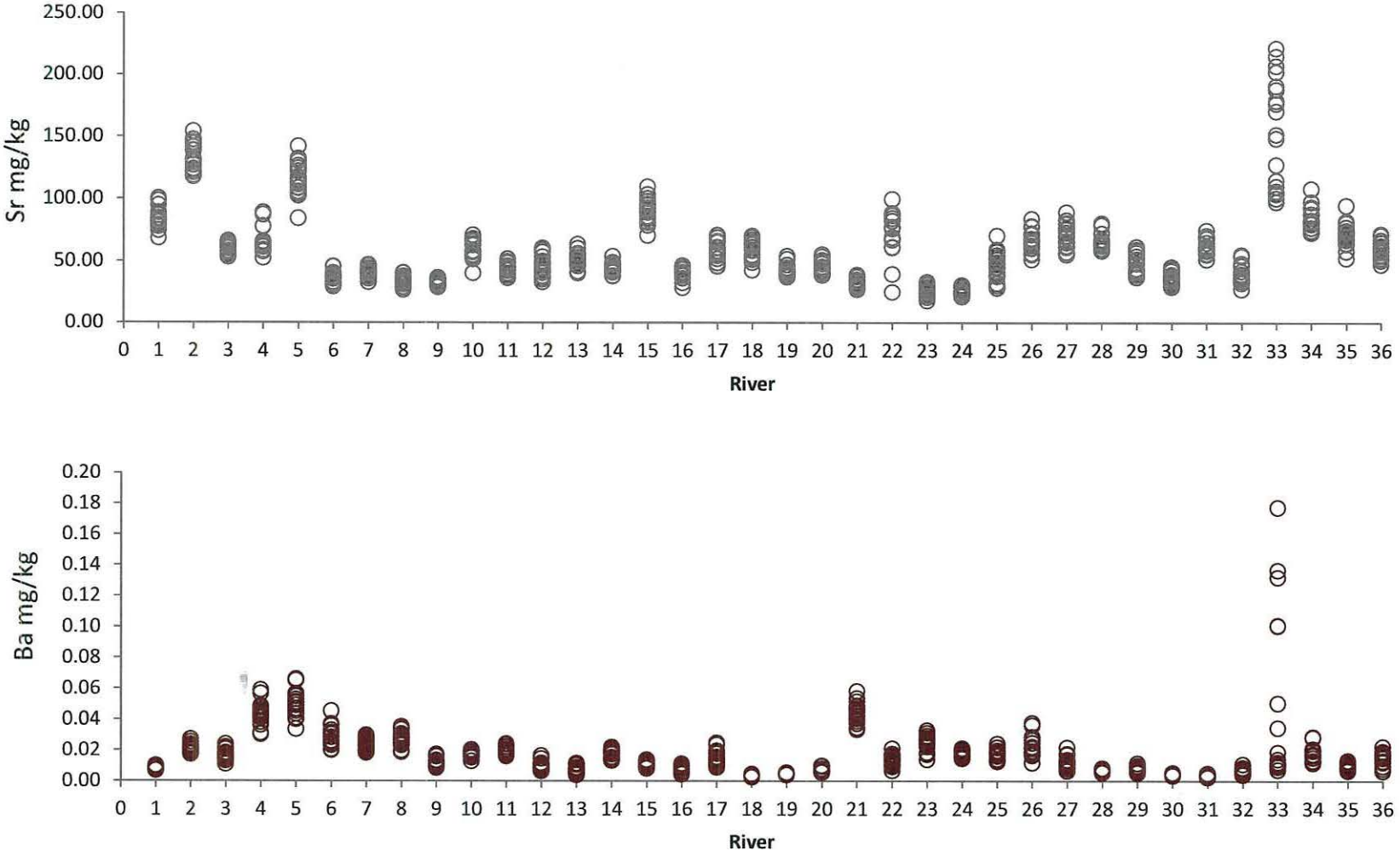
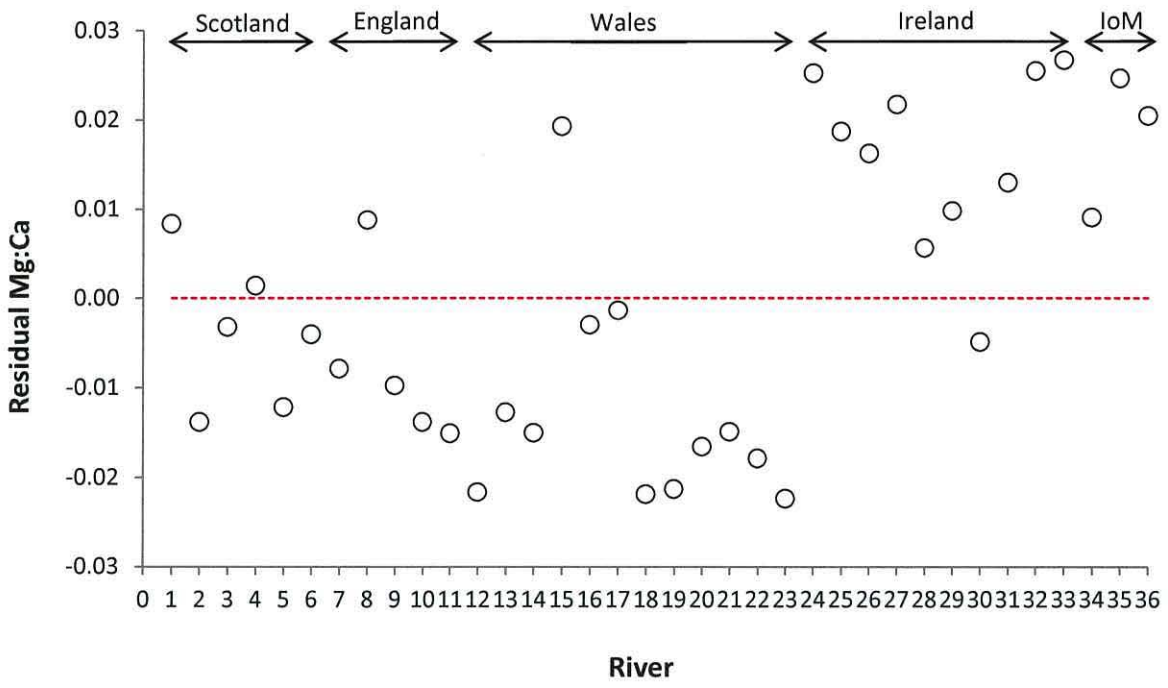
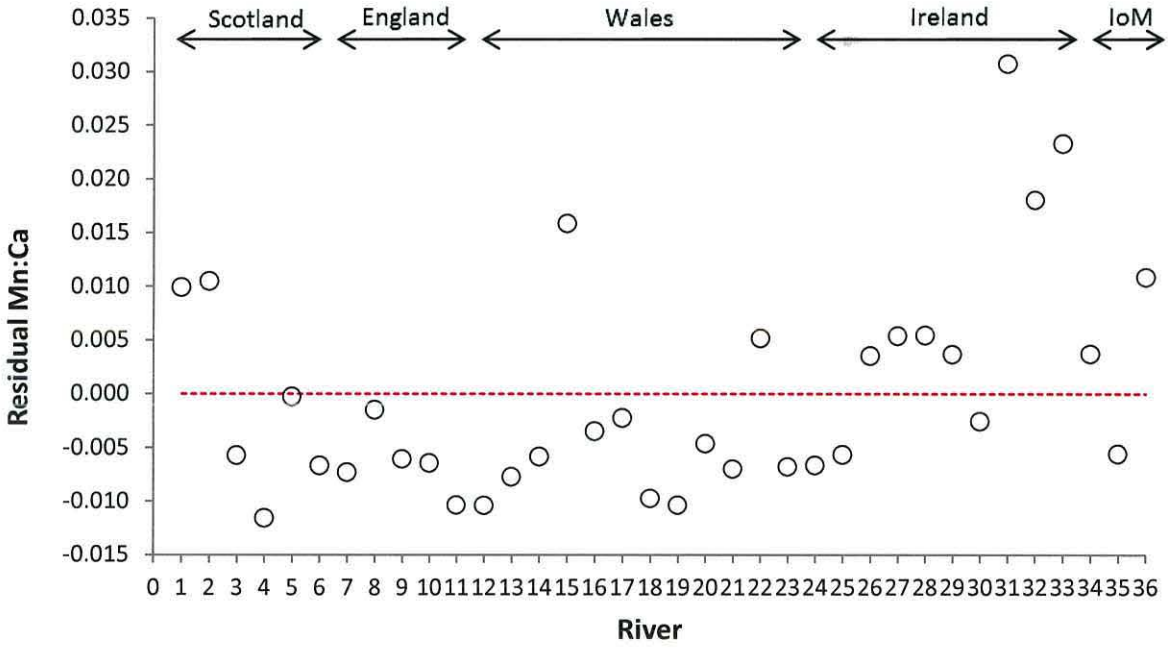


Figure 4.5. Scatter plots showing the concentrations of Mg, Mn, Sr and Ba expressed as element concentrations (mg/kg) for the juvenile *Salmo trutta* parr sampled from 36 rivers in the Irish Sea region. For river codes see Table 4.2.

Table 4.6. Element concentrations (mg/kg) for Mg, Mn, Sr and Ba in the otoliths from juvenile *Salmo trutta* parr sampled from 36 rivers located in the Irish and Celtic Sea regions. Data are presented as mean ±1 standard deviation.

Region and River	Mg	Mn	Sr	Ba
S-W Scotland				
Luce	3.409 ±0.688	1.252 ±0.175	83.920 ±7.635	0.392 ±0.051
Cree	2.478 ±0.287	1.354 ±0.433	133.463 ±11.298	1.058 ±0.109
Fleet	2.997 ±0.666	0.519 ±0.147	60.022 ±3.967	0.848 ±0.167
Nith	3.059 ±0.532	0.216 ±0.095	65.636 ±9.816	2.051 ±0.387
Annan	2.544 ±0.612	0.789 ±0.186	115.412 ±13.489	2.493 ±0.447
Border Esk	2.820 ±1.183	0.452 ±0.220	35.887 ±4.953	1.403 ±0.393
N-W England				
Ehen	2.677 ±0.622	0.426 ±0.118	40.239 ±3.870	1.170 ±0.196
Derwent	3.434 ±1.311	0.705 ±0.309	32.979 ±4.215	1.344 ±0.271
Kent	2.492 ±0.697	0.473 ±0.187	32.644 ±2.326	0.565 ±0.115
Lune	2.311 ±0.508	0.455 ±0.130	58.733 ±7.756	0.806 ±0.125
Ribble	2.369 ±0.454	0.277 ±0.070	43.427 ±4.877	1.003 ±0.156
Wales				
Dee	1.969 ±0.390	0.274 ±0.085	45.139 ±8.017	0.476 ±0.124
Clwyd	2.324 ±0.454	0.394 ±0.117	51.386 ±6.066	0.315 ±0.084
Conwy	2.300 ±0.441	0.491 ±0.174	44.632 ±3.932	0.843 ±0.143
Llyfni	3.986 ±1.257	1.564 ±0.478	90.122 ±9.482	0.527 ±0.088
Mawddach	2.827 ±0.641	0.601 ±0.167	39.347 ±4.388	0.339 ±0.094
Dyfi	3.088 ±0.631	0.697 ±0.132	57.466 ±6.969	0.724 ±0.219
Rheidol	2.016 ±0.472	0.310 ±0.097	58.021 ±7.525	0.174 ±0.027
Teifi	1.970 ±0.439	0.273 ±0.092	42.143 ±4.619	0.220 ±0.022
W. Cleddau	2.242 ±0.772	0.553 ±0.229	45.265 ±5.426	0.361 ±0.074
Tywi	2.339 ±0.683	0.446 ±0.112	31.371 ±3.407	2.181 ±0.348
Loughor	2.223 ±0.546	1.051 ±0.427	72.005 ±18.965	0.689 ±0.190
Tawe	1.900 ±0.446	0.413 ±0.089	25.401 ±4.154	1.175 ±0.247
Ireland				
Dee (White River)	4.148 ±0.950	0.445 ±0.167	25.345 ±2.559	0.826 ±0.095
Castletown	3.896 ±0.670	0.501 ±0.217	46.337 ±10.490	0.854v0.182
Dargle	4.017 ±0.928	0.987 ±0.311	66.921 ±8.686	1.124 ±0.308
Avoca	3.960 ±0.712	1.004 ±0.300	69.656 ±9.909	0.559 ±0.186
Sow	3.363 ±0.605	1.062 ±0.302	66.677 ±6.225	0.336 ±0.051
Mahon	3.499 ±0.655	0.948 ±0.238	46.643 ±7.817	0.381 ±0.089
Colligan	2.745 ±0.493	0.641 ±0.224	35.192 ±4.965	0.194 ±0.024
Argideen	3.706 ±0.398	2.286 ±0.852	61.561 ±6.535	0.176 ±0.040
Bandon	4.606 ±0.528	1.786 ±0.717	40.042 ±7.769	0.334 ±0.077
Currane	4.323 ±0.851	1.918 ±1.560	151.542 ±44.201	2.067 ±2.462
Isle of Man				
Sulby	3.452 ±1.365	0.965 ±0.727	86.737 ±9.771	0.819 ±0.272
Glass	4.184 ±1.096	0.502 ±0.133	71.988 ±9.097	0.460 ±0.102
Neb	3.935 ±0.969	1.281 ±0.408	58.756 ±6.898	0.722 ±0.211





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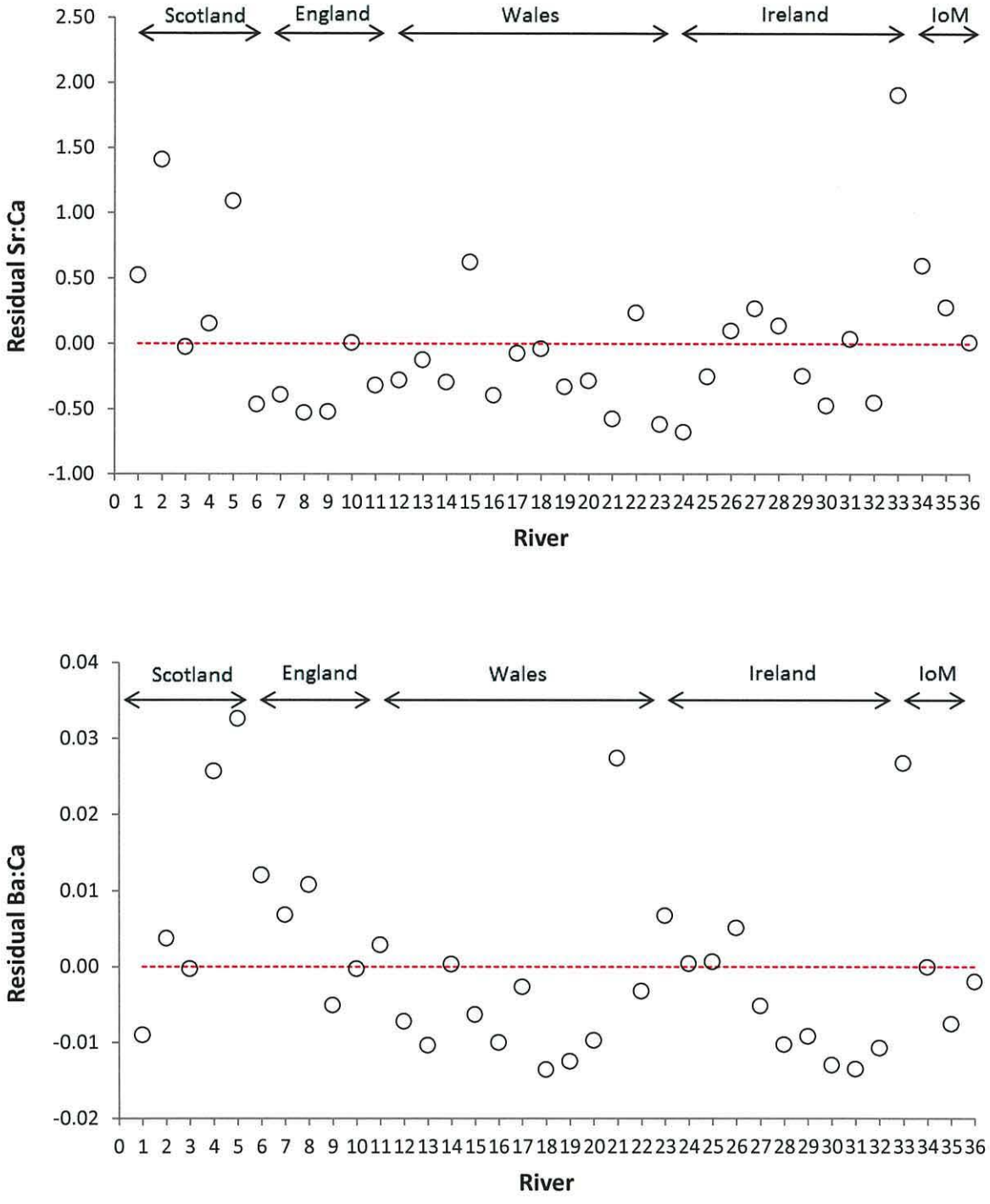


Figure 4.6. Scatter plots showing the residual concentrations of magnesium (Mg), manganese (Mn), strontium (Sr) and barium (Ba) for the juvenile *Salmo trutta* parr sampled from 36 rivers in the Irish Sea. Data for each river are expressed as a residual from the overall average element:Ca ratio for all 36 rivers combined. Rivers lying above the horizontal line have higher average element:Ca ratio and those lying below the line have lower average element:Ca ratio than the average for all 36 rivers combined. For river codes see Table 4.2. (IoM = Isle of Man).

Using QDFA, with river as the variable and the four elements Mg, Mn, Sr and Ba as predictors, original classification of juvenile brown trout parr to their river of origin was high at 84.2%, with 560/665 parr correctly classified back to their natal river (see Table 4.7.). Similarly, using cross-validation (leave one out method), correct classification was also high at 73.7% with 490/665 juvenile brown trout parr correctly classified back to their river of origin (Table 4.8.). Chance-corrected classification accuracy using Cohen's kappa statistic was 0.84 (± C.I 0.813 - 0.871) for QDFA and 0.73 (± C.I 0.694 - 0.764) for CV-QDFA respectively.

Classification accuracy varied between rivers with assignments using CV-QDFA ranging between 42% and 100% correct classification (see Figure 4.7.), with the majority of the classifications greater than 70% (21/36). Four rivers fell below 50% correct classification: Dyfi (42%), Fleet and Mahon (45%) and Avoca (47%). However, five rivers reported 100% assignment – Nith, Annan, Tywi, Argideen and Currane and a further five rivers exceeded 85% correct classification: Cree (95%), Rheidol (95%), Teifi (89%), Dee (White River - 87%) and Bandon (89%) (Table 4.8., Figure 4.7.). Some trout parr that did not classify back to their river of origin were classified to a geographically-adjacent river: usually 1-2 individuals (5-13% of a data set) classified to an adjacent river/s (see Table 4.7 and Table 4.8.).

Using principal component analysis (PCA) the standard correlation matrix indicated Mn and Ba were the most significant elements in explaining the variation and discrimination between the 36 rivers with principal component loadings of 0.569 and -0.055 respectively (Figure 4.8.). PCA-1 indicated a variance (eigenvalue) of 1.787 and accounted for 44.7 % of the total variance, with PCA-2 eigenvalue of 1.046 accounting for 26.2 % of the variability. Combined, the first two principal components (PCA-1 and PCA-2) represented 70.8 % of the combined total variability observed for the 36 rivers using the otolith trace elements Mg, Mn, Sr and Ba whilst the first three principal components (PCA-1, PCA-2 and PCA-3) representing 90 % of the total variability.

To assess the impact of minor deviations in normality and homoscedasity within the dataset on the QDFA classification results, individual parr were also assigned to a river of origin using Random Forest to compare the results observed using the original QDFA.

Table 4.7. Quadratic discriminant function analysis (QDFA) classification of *S. trutta* parr to each of the 36 rivers of capture using the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba. Highlighted areas correspond to those parr classified to adjacent rivers. Correct number of parr to river highlighted in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1.Luce	**17**														4																						
2.Cree		**19**																																		
3.Fleet			**12**							1		2					3									2										
4.Nith				**19**																																
5.Annan					**19**																															
6.Border Esk						**13**	1	2																												
7.Ehen						2	**17**				2																									
8.Derwent						3		**16**															1													
9.Kent									**19**																											
10.Lune			3							**11**																1										
11.Ribble							1				**15**	1		2																						
12. Dee			2									**16**	2							1																
13.Clwyd													**14**						1																	
14.Conwy							1				2			**16**											1											
15.Llyfni	2														**12**																					
16.Mawddach																**16**	2			1									3	1		1				
17.Dyfi			1							1							**11**			1		1														
18.Rheidol																		**20**																		
19.Teifi																2			**17**	1										2						
20.W. Cleddau													1				1			**13**		1							1							
21.Tywi																					**20**															
22.Loughor										1			1									**11**	1			1										1
23.Tawe								1														1	**17**													
24.Dee (W.R)																								**15**												
25.Castletown																									**16**		1									
26.Dargle			2							1							2								2	**15**										5
27.Avoca																											**14**		1							
28. Sow															1					1							1	**15**								2
29.Mahon																				1							1		**12**			1				1
30.Colligan													1			1														**15**						
31.Argideen																															**19**					
32.Bandon																1																**17**				
33.Currane																																	**20**			
34.Sulby																						1					1							**14**		
35.Glass																																			**19**	
36 Neb																						1				1			3					1		**9**
Total N°	19	19	20	19	19	18	20	19	19	15	19	19	19	18	17	20	19	20	18	19	20	16	19	15	19	19	19	15	20	18	19	19	20	15	19	18
N° correct	17	19	12	19	19	13	17	16	19	11	15	16	14	16	12	16	11	20	17	13	20	11	17	15	16	15	14	15	12	15	19	17	20	14	19	9
Proportion (%)	90	100	60	100	100	72	85	84	100	73	79	84	74	89	71	80	58	100	94	68	100	69	90	100	84	79	74	100	60	83	100	90	100	93	100	50

Table 4.8. Cross-validation quadratic discriminant function analysis (CV-QDFA) classification of *S. trutta* parr to each of the 36 rivers of capture using the Log*10* elemental concentrations of Mg, Mn, Sr and Ba. Highlighted areas correspond to those parr classified to adjacent river. Correct number of parr to river highlighted in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1.Luce	**15**														4																					
2.Cree		**18**																																		
3.Fleet			**9**							2		2					3								2	2										
4.Nith				**19**																																
5.Annan					**19**																															
6.Border Esk						**10**	2	4																												
7.Ehen						2	**14**				4																									
8.Derwent						6		**13**	1														2	1												
9.Kent									**15**																											
10.Lune			3							**10**							1									1										
11.Ribble							2		1		**13**	1		2																						
12. Dee			2						1			**15**	2						1	1																
13.Clwyd			1									1	**12**					1	1									1		1				2		
14.Conwy							1				2			13											1											
15.Llyfni	2														11																					
16.Mawddach																**12**	2			1						1			5	1		1			
17.Dyfi			3							1						1	**8**			1		1				1									
18.Rheidol																		**19**																	
19.Teifi																2			**16**	1										2					
20.W. Cleddau													2			1	1			**12**		1							1							
21.Tywi																					**20**		1													
22.Loughor	1	1					1			1			1	3								**9**	**1**				1						1		1	
23.Tawe								2														1	**15**													
24.Dee (W.R)																								**13**												
25.Castletown									1															1	**14**		1									
26.Dargle			2							1							3								2	**11**										5
27.Avoca	1														1												**9**	1	1				1	2		
28. Sow															1					1							2	**12**							2	
29.Mahon																2				1							2		**9**			1			1	
30.Colligan													1			1														**14**						
31.Argideen																															**19**					
32.Bandon																1													1			**17**				
33.Currane																										1							**20**	1		
34.Sulby																						3					2							**11**		
35.Glass													1														1								**15**	
36 Neb																	1					1				2	1	1	3					1		**9**
Total N°	19	19	20	19	19	18	20	19	19	15	19	19	19	18	17	20	19	20	18	19	20	16	19	15	19	19	19	15	20	18	19	19	20	15	19	18
N° correct	15	18	9	19	19	10	14	13	15	10	13	15	12	13	11	12	8	19	16	12	20	9	15	13	14	11	9	12	9	14	19	17	20	11	15	9
Proportion (%)	79	95	45	10	100	56	70	68	79	67	68	79	63	72	65	60	42	95	89	63	100	56	79	87	74	58	47	80	45	78	100	90	100	73	79	50

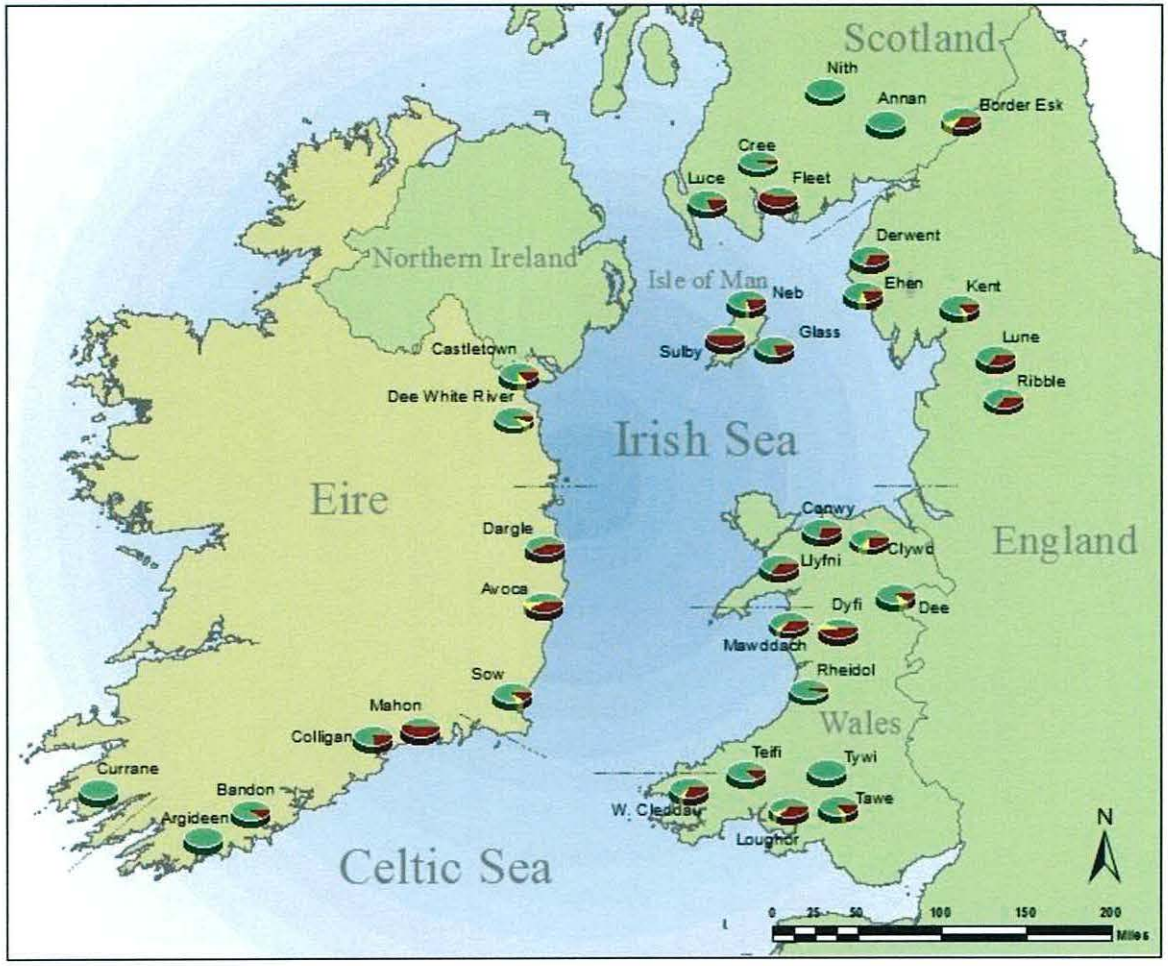


Figure 4.7. Map of the Irish and Celtic Sea region indicating classification of *S. trutta* parr to each of the 36 rivers of capture using cross-validation QDFA from Mg, Mn, Sr and Ba. Pie chart: **Green**- correct classification; **yellow** – adjacent river; **red** – misclassified river.

Classification rates of trout parr back to river of origin based on their Mg, Mn, Sr and Ba otolith chemistry using Random Forest were observed to be slightly lower with an overall classification rate of 70.8%, and with 471 out of 665 juvenile trout parr correctly classifying back their natal river (Table 4.9.). The chance-corrected Random Forrest classification accuracy using Cohen's kappa statistic was similar to the CV-QDFA at 0.70 (± C.I 0.664 - 0.736).

Results using Random Forest analysis were similar to those observed for CV-QDFA, with correct classification of individual trout parr back to their river of origin ranging between 26% - 100% (Table 4.9.) but with the classification success being river-dependent. There was an increase in the number of rivers exhibiting less than 50% classification from 4 using CV-QDFA to five using Random Forest: Avoca (26%), Fleet (33%), Dyfi (37%), Border Esk (39%) and the river Dargle (47%). Furthermore, there was a reduction in rivers indicating classification success of 100% from five using CV-QDFA to two: the Cree and Argideen.

However, a greater number of rivers (10 compared to five using CV-QDFA) indicated correct classifications in excess of 85%: Currane (85%), Teifi, Colligan (89%), Luce, Rheidol, Tywi (90%), Dee (White River – 93%) and Nith, Annan and Glass (95%). As with the DFA analysis, some trout parr that did not classify back to their river of origin were classified to a geographically-adjacent river. Using Random Forest classification of trout parr to adjacent rivers ranged between 1-4 individuals (5-22%) dependent on river/s and are highlighted (see Table 4. 9.).

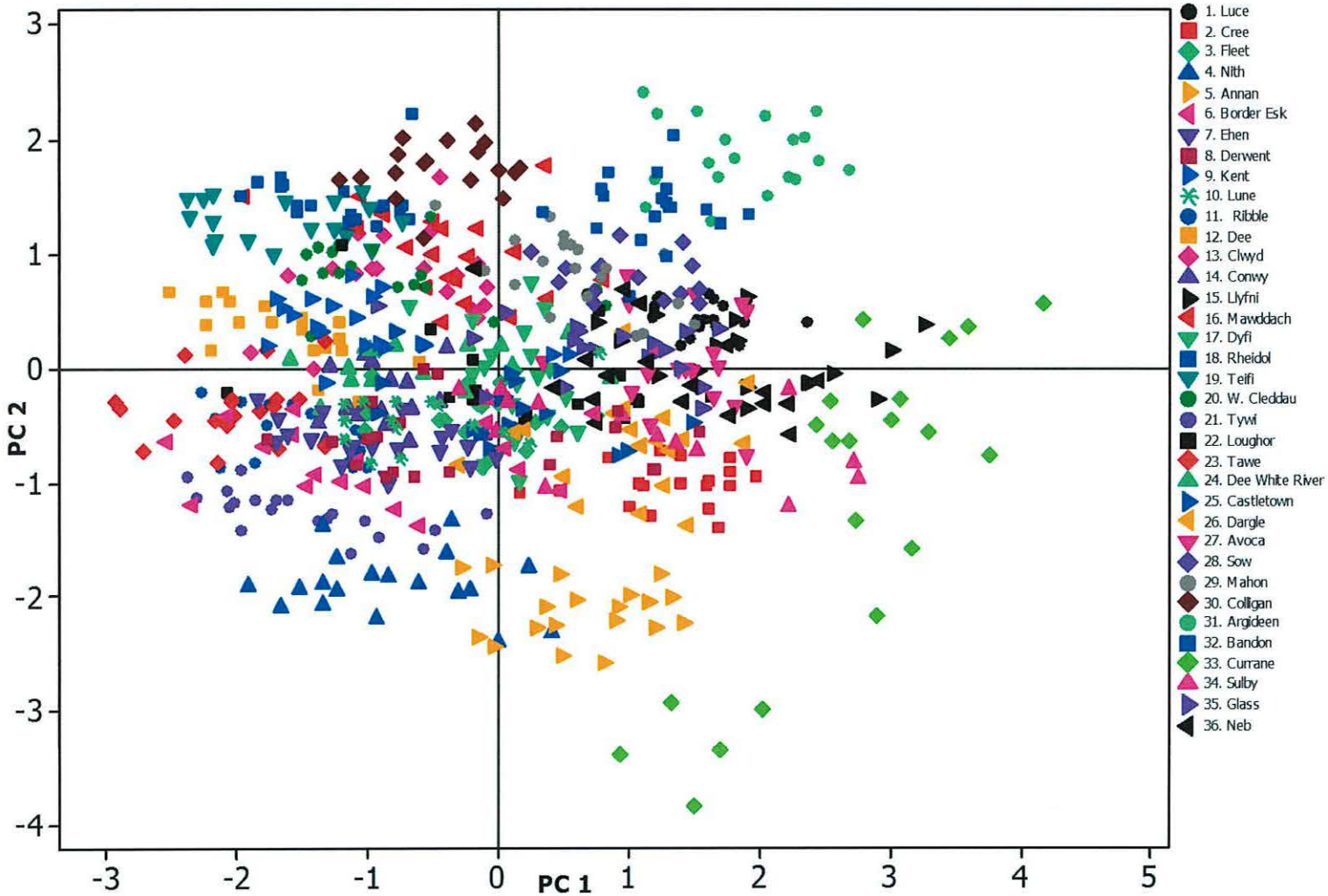


Figure 4.8. PCA indicating the classification groupings of juvenile trout parr (*Salmo trutta*) for the 36 rivers sampled based on sagittal otolith microchemistry from the elements Mg, Mn, Sr and Ba.

Table 4.9. Random Forest (RF) classification of *S. trutta* parr to each of the 36 rivers of capture using the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba. Highlighted areas correspond to those parr classified to adjacent rivers. Correct number of parr to river highlighted in bold.

	5	31	27	32	25	13	30	14	2	33	26	12	24	8	17	7	6	3	35	9	15	22	1	10	29	16	36	4	18	11	28	34	23	19	21	20	N°	N° Correct	Proportion (%)
5.Annan	**18**									1																													19	18	94.7
31.Argideen		**19**																																					19	19	100
27.Avoca			**5**								2				1				1		3				2		2				2	1						19	5	26.3
32.Bandon				**16**			1																		2													19	16	84.2
25.Castletown					**11**			2			2							2		2																			19	11	57.9
13.Clwyd						**10**	1					3			1														1							3	19	10	52.6
30.Colligan							**16**																															18	16	88.9
14.Conwy								**15**																						3				2				18	15	83.3
2.Cree									**19**																													19	19	100
33.Currane	2									**17**											1																	20	17	85
26.Dargle	1										**9**				3			1						1	1		3											19	9	47.4
12.Dee						1						**15**						1						1						1								19	15	78.9
24.Dee W.R													**14**	1																								15	14	93.3
8.Derwent													1	**12**			4																	1		1		19	12	63.2
17.Dyfi					1						1				**7**			6				1			2											1	19	7	36.8
7.Ehen					1			1						1		**12**														5								20	12	60
6.Border Esk														5		4	**7**																		2		18	7	38.9
3.Fleet					2	1					1				5			**6**						5								1					20	6	30
35.Glass																			**18**																		19	18	94.7
9.Kent												1		2		1				**14**						1											19	14	73.7
15.Llyfni			1							1											**12**		2							1							17	12	70.6
22.Loughor			1												1			1			2	**9**											1			1	16	9	56.3
1.Luce																					2		**17**														19	17	89.5
10.Lune					1										1			3				1		**9**													15	9	60
29.Mahon			1	2			1																		**11**	4	1										20	11	55
16.Mawddach							1								1										2	**11**								2		3	20	11	55
36.Neb			1								3											1			1		**10**				2						18	10	55.6
4.Nith																		1										**18**									19	18	94.7
18.Rheidol							1																						**18**					1			20	18	90
11.Ribble								3								2														**14**							19	14	73.7
28.Sow			1			1													1												**12**						15	12	80
34.Sulby									1	1											3						1					**9**					15	9	60
23.Tawe																	2			1													**16**				19	16	84.2
19.Teifi												1																	1					**16**			18	16	88.9
21. Tywi																																	2		**18**		20	18	90
20.W. Cleddau						1						1			1										2	1					1			1		**11**	19	11	57.9

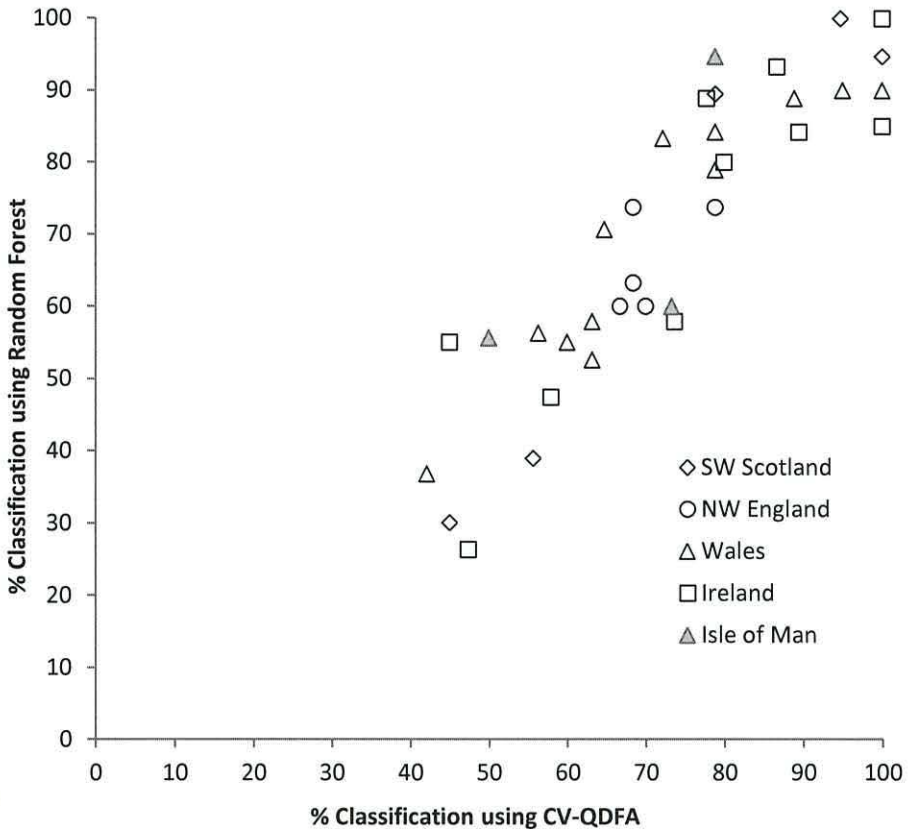


Figure 4.9. Scatterplot showing the relationship between classification accuracies assigning juvenile *Salmo trutta* parr back to their natal river using cross-validated Quadratic Discriminant Function analysis (CV-QDFA) and Random Forest analysis.

Classification accuracies using CV-QDFA and Random Forest were significantly correlated (r_{36} = 0.898, P < 0.001; Figure 4.9.) and described by the following linear regression:

$$Y = 1.05X - 6.51 \ (r^2 = 0.806, P < 0.001)$$

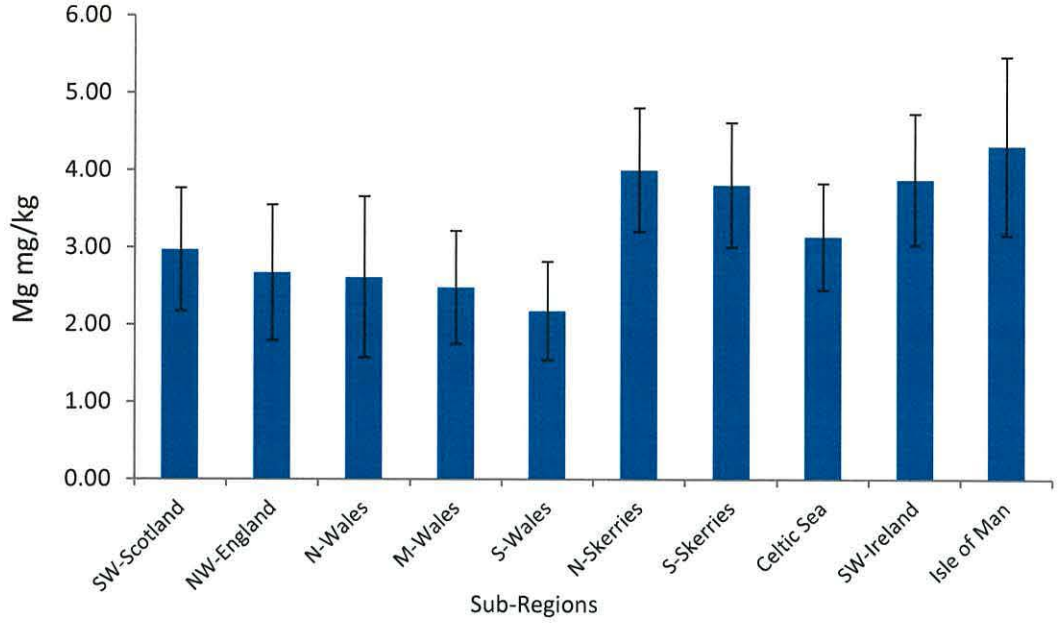
where Y is the % classification success using Random Forest and X is the % classification success using CV-QDFA. The intercept (-6.51 ± 6.69 se) for the regression line was not significantly different from zero (t = -0.97, P = 0.34), with the forced regression line indicating a slope of 0.97 (± 0.02 se).

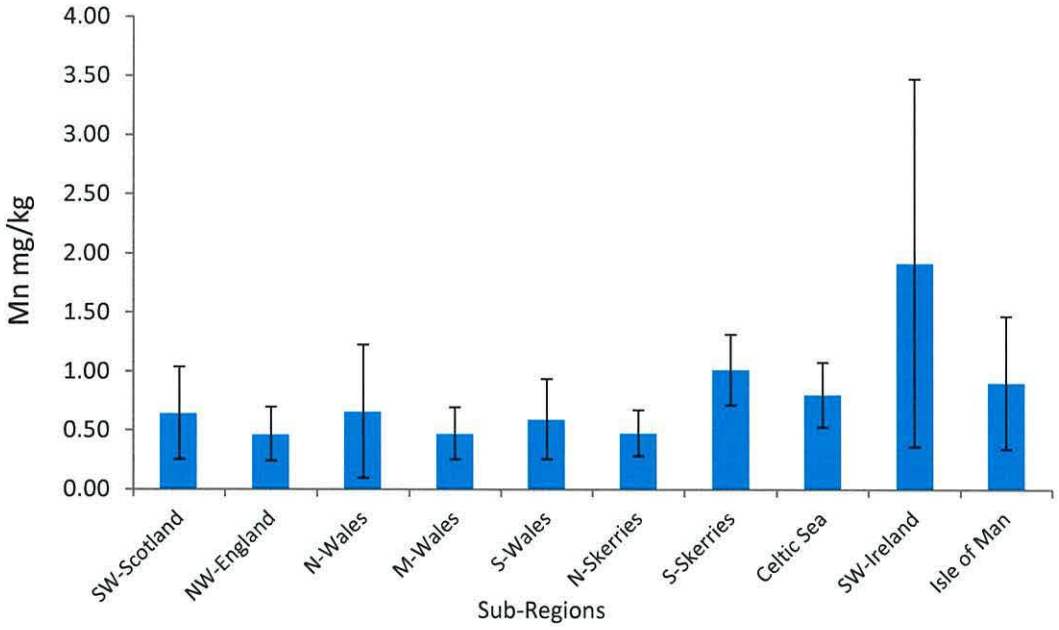
4.5.3. Classification of parr to their sub-region of origin

The data for the concentrations of Mg, Mn, Sr and Ba (expressed as mg/kg) in the sagittal otoliths of the juvenile trout parr were grouped according to sub-region (Figure 4.3.) and the mean (± sd) values are presented in Figure 4.10. and Table 4.10. respectively. Visual assessment of the elemental concentrations for each of the 10 sub-regions indicated Mg and Mn had lower concentrations for the rivers sampled from the five UK sub-regions located in SW-Scotland, NW-England, and Wales compared to rivers sampled from the 5 sub-regions located in Ireland and the Isle of Man (see Figure 4.10. and Table 4.10.). The most notable difference was for SW-Ireland which presented higher concentrations for both Sr and Ba when compared to the other 9 sub-regions (Table 4.10.) as a result of the data for the River Currane (see Table 4.6.).

Table 4.10. Concentrations of Mg, Mn, Sr and Ba (expressed as mg/kg) in otoliths from juvenile trout parr (*Salmo trutta*) sampled in the 10 sub-regions around the Irish and Celtic Seas. River No's refer to the number of sampled rivers located within each of the 10 sub-regions. Data are presented as mean ±1 standard deviation.

Region	River N°'s	Mg	Mn	Sr	Ba
SW-Scotland	6	2.968 ± 0.797	0.646 ± 0.392	72.430 ± 27.748	1.432 ± 0.835
NW-England	5	2.672 ± 0.875	0.467 ± 0.228	40.845 ± 10.142	0.987 ± 0.330
N-Wales	4	2.613 ± 1.043	0.659 ± 0.567	57.116 ± 19.828	0.537 ± 0.223
M-Wales	4	2.481 ± 0.734	0.472 ± 0.221	49.332 ± 10.494	0.363 ± 0.247
S-Wales	4	2.176 ± 0.639	0.596 ± 0.341	42.191 ± 19.778	1.133 ± 0.745
N-Skerries	2	4.007 ± 0.803	0.477 ± 0.195	37.079 ± 13.219	0.842 ± 0.149
S-Skerries	3	3.811 ± 0.808	1.014 ± 0.300	67.832 ± 8.511	0.699 ± 0.396
Celtic Sea	4	3.141 ± 0.691	0.803 ± 0.276	41.219 ± 8.734	0.293 ± 0.115
SW-Ireland	1	4.323 ± 0.851	1.918 ± 1.560	151.542 ± 44.201	2.067 ± 2.462
Isle of Man	3	3.887 ± 1.157	0.905 ± 0.564	71.662 ± 14.034	0.654 ± 0.250





(Figure 4.10. and caption are continued overleaf)

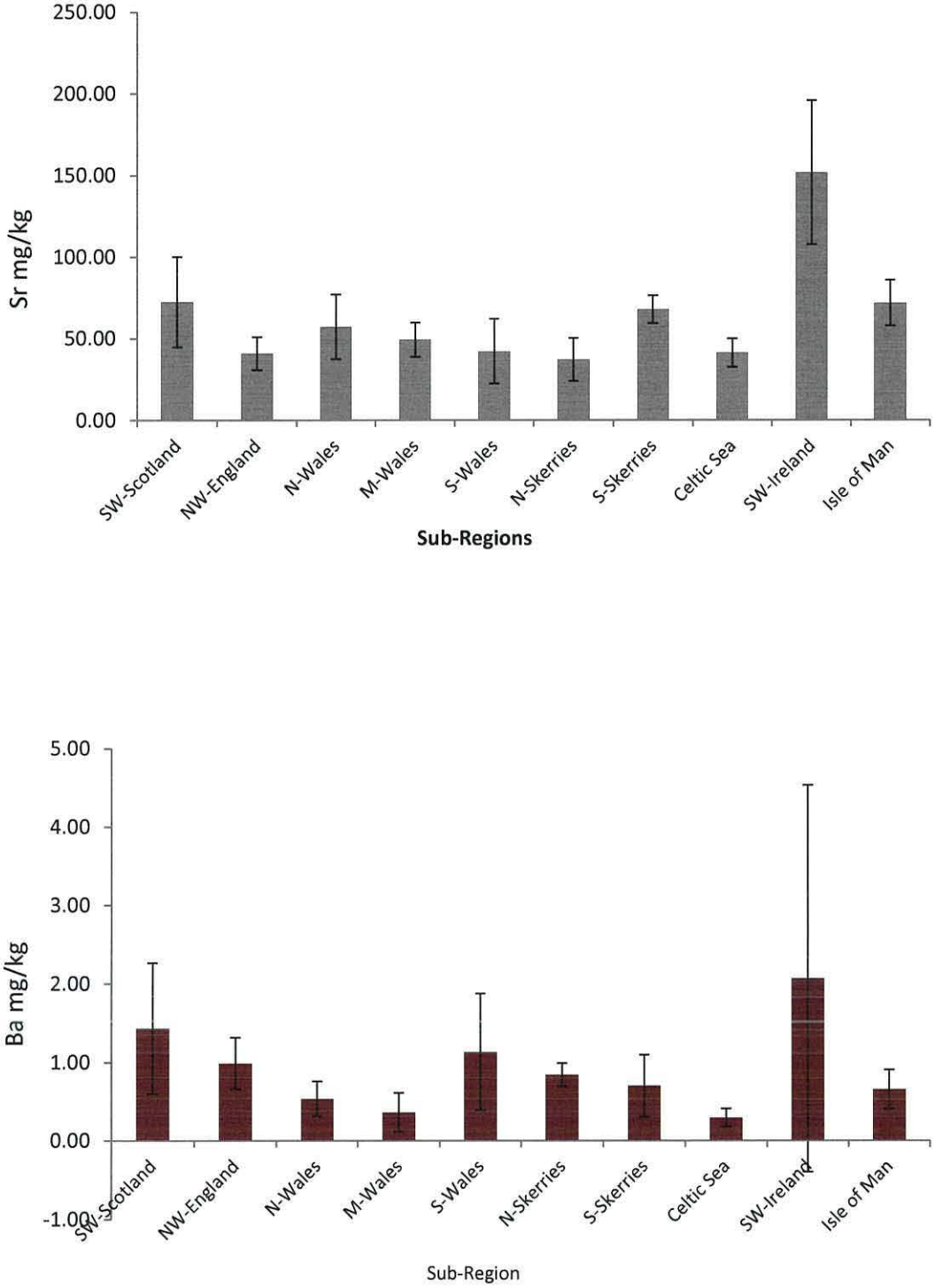


Figure 4.10. Differences in element concentrations for Mg, Mn, Sr and Ba in the otoliths of juvenile *Salmo trutta* parr sampled from 10 sub-regions in the Irish and Celtic Seas. See Figure 4.3. for a map to identify sub-regions.

Significant differences were observed for the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba in the otoliths of trout parr between the 10 sub-regions (MANOVA: using Willks' criterion: $F_{36, 2445} = 54.120$, $P < 0.001$). ANOVAs conducted separately on each of the four elements - Mg, Mn, Sr and Ba - indicated highly significant differences in elemental concentrations between the 10 sub-regions (Mg: $F_{9, 664} = 51.89$, $P < 0.001$; Mn: $F_{9, 664} = 33.54$, $P < 0.001$; Sr: $F_{9, 664} = 81.08$, $P < 0.001$; Ba: $F_{9, 664} = 71.58$, $P < 0.001$).

Post-hoc multiple pairwise comparisons (using the Bonferroni test) indicated significant ($P < 0.05$) or highly significant ($P < 0.001$) differences between sub-regions with respect to Mg, Mn, Sr and Ba otolith concentrations (see Table 4.11.). Mg concentrations differed significantly between sub-regions for 29/45 pairwise comparisons (*i.e.* 64%). For Mg, all pairwise comparisons between the Irish and Isle of Man sub-regions and the UK sub-regions were significantly different and S Wales was different to N Wales, NW England and SW Scotland (see Table 4.11.). Similarly, Mn also exhibited trans-Irish Sea differences in otolith chemistry with 27/45 pairwise comparisons (*i.e.* 60%) significantly different (see Table 4.11.). Mn concentrations in the UK and North Skerries sub-regions were different to those reported for the remaining Irish and Isle of Man sub-regions (see Table 4.11.). In contrast, concentrations of the elements Sr and Ba in *S. trutta* otoliths was more variable with 35/45 (*i.e.* 77%) pairwise comparisons between sub-regions being significantly different for Sr and 23/45 (*i.e.* 51%) pairwise comparisons being significantly different between the 10 sub-regions for Ba (see Table 4.11.). For both Sr and Ba, there were no apparent geographical patterns in the significant differences observed between sub-regions.

Principal component analysis (PCA) using the standard correlation matrix observed for classification of the 36 rivers using the elements Mg, Mn, Sr and Ba (see Figure 4.8.) was used to identify group clustering's for each sub-region (Figure 4.11.). Classification using the regions as the grouping for the score plot indicated a good degree of separation between each of the 10 sub-regions based on the otolith chemistry of the juvenile trout parr (Figure 4.11.). Score-plot groupings were good, the most notable of which were the sub-regions North, Mid and South Wales and the North-West England which grouped together. Four regions however, SW-Scotland, SW-Ireland, Celtic Sea and the Isle of Man tended to cluster as discrete groups (Figure 4.11.). Similar observations of discrete groupings could also be observed for the North and South Skerries sub-regions.

Table 4.11. Results of *post-hoc* comparisons (using a Bonferroni test) between the 10 sub-regions to determine where significant differences in otolith chemistry (element:Ca ratios) are following a significant ANOVA. Significant differences are at the α 0.05 level (*) or < 0.001 (**).

Mg

Region	1	2	3	4	5	6	7	8	9	10
1 SW Scotland										
2 NW England	1.00									
3 N. Wales	0.29	1.00								
4 Mid Wales	*	1.00	1.00							
5 S Wales	**	**	*	0.13						
6 N. Skerries	**	**	**	**	**					
7 S Skerries	**	**	**	**	**	1.00				
8 Celtic Sea	**	**	**	**	**	0.47	1.00			
9 SW Ireland	**	**	**	**	**	1.00	1.00	0.11		
10 Isle of Man	**	**	**	**	**	1.00	1.00	1.00	1.00	

Mn

Region	1	2	3	4	5	6	7	8	9	10
1 SW Scotland										
2 NW England	*									
3 N. Wales	1.00	1.00								
4 Mid Wales	*	1.00	1.00							
5 S Wales	1.00	0.68	1.00	0.42						
6 N. Skerries	0.44	1.00	1.00	1.00	1.00					
7 S Skerries	**	**	**	**	**	**				
8 Celtic Sea	**	**	**	**	**	**	1.00			
9 SW Ireland	**	**	**	**	**	**	0.11	1.00		
10 Isle of Man	0.24	**	**	**	*	*	1.00	*	*	

Table 4.11. Continued

Sr

Region	1	2	3	4	5	6	7	8	9	10
1 SW Scotland										
2 NW England	**									
3 N. Wales	**	**								
4 Mid Wales	**	*	0.29							
5 S Wales	**	1.00	**	*						
6 N. Skerries	**	1.00	**	*	1.00					
7 S Skerries	1.00	**	*	**	**	**				
8 Celtic Sea	**	1.00	*	1.00	0.22	*	**			
9 SW Ireland	**	**	**	**	**	**	**	**		
10 Isle of Man	1.00	**	*	**	**	**	1.00	**	**	

Ba

Region	1	2	3	4	5	6	7	8	9	10
1 SW Scotland										
2 NW England	0.56									
3 N. Wales	**	**								
4 Mid Wales	**	**	**							
5 S Wales	0.13	1.00	**	**						
6 N. Skerries	0.25	1.00	*	**	1.00					
7 S Skerries	**	*	1.00	**	*	0.14				
8 Celtic Sea	**	**	**	1.00	**	**	**			
9 SW Ireland	1.00	1.00	**	**	1.00	1.00	*	**		
10 Isle of Man	**	*	1.00	**	*	0.38	1.00	**	*	

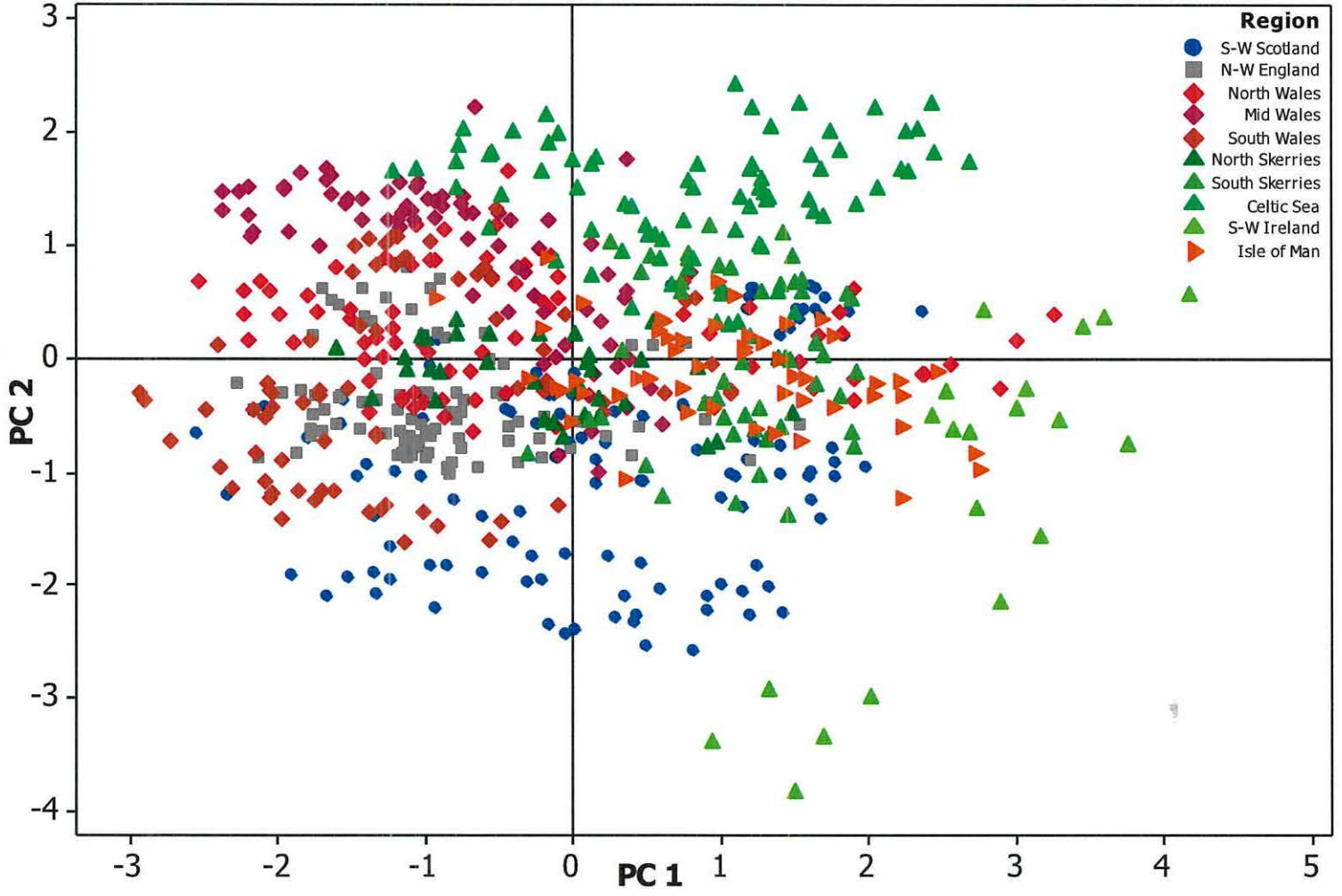


Figure 4.11. PCA indicating the classification groupings of juvenile trout parr (*Salmo trutta*) for the 10 sub-regions sampled based on sagittal otolith microchemistry from the elements Mg, Mn, Sr and Ba.

When fish were grouped according to the 10 sub-regional groupings, classifications accuracy using QDFA was 68.7%, with 457/665 trout parr correctly classified back to their sub-regional of origin (Table 4.12.). Cross-validation QDFA classification accuracy indicated similar results with 66.2% (*i.e.* 440/665) of trout parr correctly classified back to their correct sub-region of origin (Figure 4.12., Table 4.13.). Classification accuracy for sub-regions using CV-QDFA ranged between 41% - 91% (Table 4.13.). Two regions indicated percentage classification accuracies equal to or lower than 50%: N-Wales (41%) and SW-Scotland (50%). Five regions showed correct trout parr classifications between 60% and 80%: S-Wales (64%), NW-England, S-Skerries and the Isle of Man (all 69%) and M-Wales (75%). Both the Celtic Sea and SW-Ireland indicated the most distinct elemental signals of the 10 sub-regions with the classification of trout parr to those sub-regions being 81% and 85% respectively, with one region N-Skerries showing a correct assignment of trout parr to region of 91% (Table 4.13.). The chance-corrected original classification accuracy of trout parr to region using Cohen's kappa statistic was 0.65 (± C.I 0.608 - 0.689) for QDFA and 0.62 (± C.I 0.578 - 0.661) for CV-QDFA respectively.

Similar to the river classification assessment, trout parr were also assigned to sub-region using Random Forest analysis as a comparison to the results observed using the original QDFA / CV-QDFA. Classification accuracy to sub-region of origin using Random Forest were higher compared to QDFA at 74.4%, with 495/665 trout parr correctly assigned to their region of origin (Table 4.14.). Chance-corrected Random Forrest classification accuracy of trout parr to region using Cohen's kappa was 0.71 (± C.I 0.674 - 0.757).

Individual parr classification to sub-region was higher using Random Forest compared to QDFA with a range between 58% - 90%. Four regions indicated correct classifications greater than 70%: Mid Wales (70%), Scotland (72%), S-Wales (73%) and N-Skerries (79%), with NW England indicating correct assignment of trout parr to region of 80%. Again, both the Celtic Sea and SW-Ireland showed distinct classification results similar to those observed using QDFA (90% and 85% respectively). However, it is noteworthy that none of the three classification techniques assigned trout parr to their sub-region of origin with 100% success.

Table 4.12. Quadratic discriminant function analysis (QDFA) classification of *S. trutta* parr to each of the 10 sub-regional zones of capture using the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba. Highlighted areas correspond to those parr classified to adjacent sub-regions. Correct number of parr to sub-region highlighted in bold.

	S-W Scotland	N-W England	North Wales	Mid Wales	South Wales	North Skerries	South Skerries	Celtic Sea	S-W Ireland	Isle of Man
S-W Scotland	**57**	2	3	3	4		2			3
N-W England	17	**67**	15		2	1				
North Wales	22	2	**31**	4	4		3			
Mid Wales		1	14	**58**	10		1	5		1
South Wales	4	13	5	1	**51**					1
North Skerries	9	5	1	5		**31**	1			
South Skerries	4	1	2	1	2	2	**39**	7		8
Celtic Sea		1	1	5			1	**64**		
S-W Ireland	1								**20**	
Isle of Man			1		1		6			**39**
N°	114	92	73	77	74	34	53	76	20	52
N° correct	57	67	31	58	51	31	39	64	20	39
Proportion (%)	50.0	72.8	42.5	75.3	68.9	91.2	73.6	84.2	100	75.0

Table 4.13. Cross-validation quadratic discriminant function analysis (CV-QDFA) classification of *S. trutta* parr to each of the 10 sub-regional zones of capture using the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba. Highlighted areas correspond to those parr classified to adjacent sub-regions. Correct number of parr to sub-region highlighted in bold.

	S-W Scotland	N-W England	North Wales	Mid Wales	South Wales	North Skerries	South Skerries	Celtic Sea	S-W Ireland	Isle of Man
S-W Scotland	**57**	2	3	3	4		3		2	4
N-W England	17	**64**	15		2	1				
North Wales	22	2	**30**	4	4		3		1	2
Mid Wales		1	14	**58**	11		1	6		1
South Wales	4	15	5	1	**48**					1
North Skerries	9	6	1	5		**31**	1			
South Skerries	4	1	2	1	2	2	**37**	8		8
Celtic Sea		1	1	5			1	**62**		
S-W Ireland	1		1						**17**	
Isle of Man			1		3		7			**36**
N°	114	92	73	77	74	34	53	76	20	52
N° correct	57	64	30	58	48	31	37	62	17	36
Proportion (%)	50.0	69.6	41.1	75.3	64.9	91.2	69.8	81.6	85.0	69.2

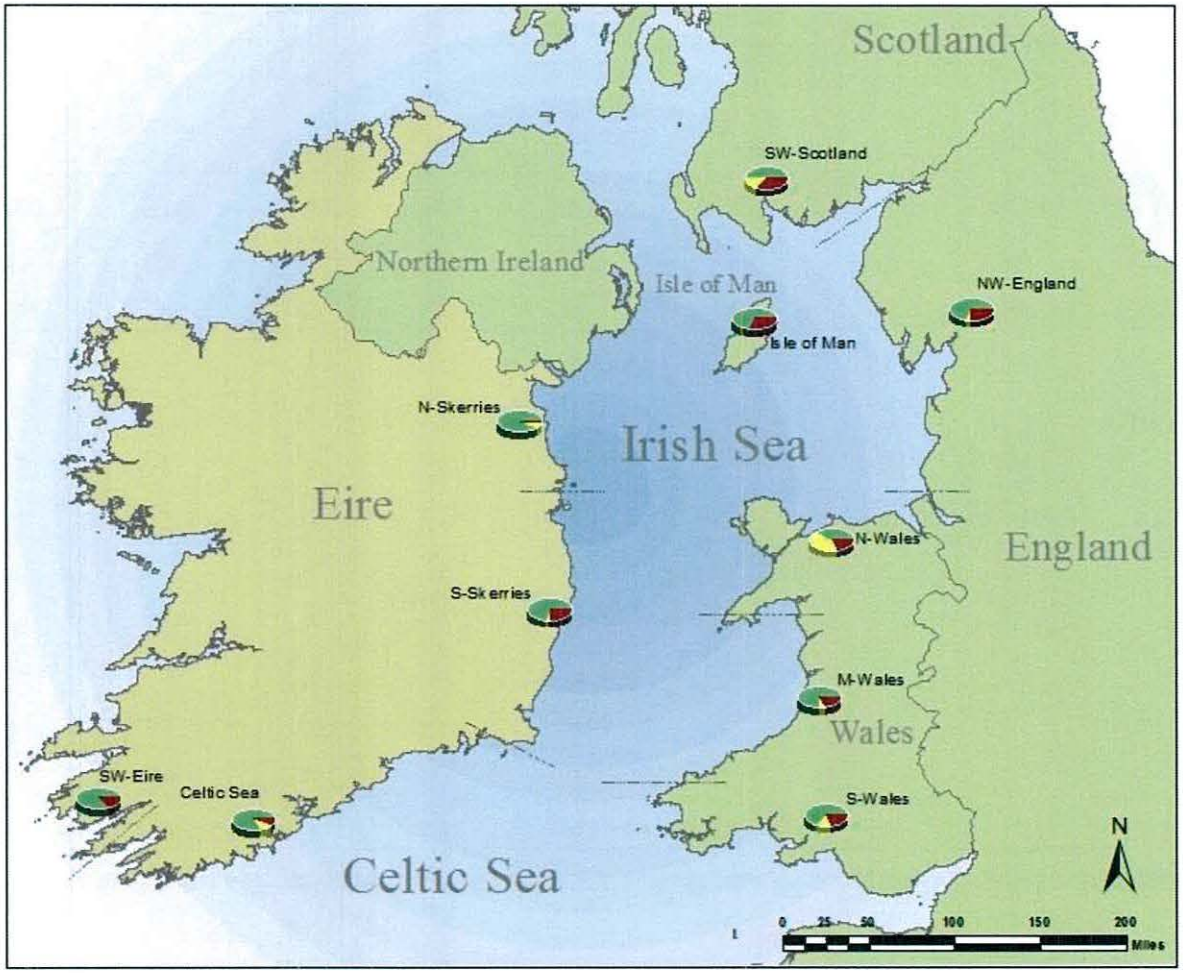


Figure 4.12. Map of the Irish and Celtic Sea region indicating classification of *S. trutta* parr to each of the 10 sub-regions of capture using cross-validation QDFA from Mg, Mn, Sr and Ba. Pie chart: Green- correct classification; yellow – adjacent river; red – misclassified river.

Table 4.14. Random Forest (RF) classification of *S. trutta* parr to each of the 10 sub-regional zones of capture using the Log_{10} elemental concentrations of Mg, Mn, Sr and Ba. Highlighted areas correspond to those parr classified to adjacent sub-regions. Correct number of parr to sub-region highlighted in bold.

	Celtic Sea	England	Isle of Man	Mid Wales	N-Skerries	N-Wales	Scotland	S-Skerries	S-Wales	SW-Ireland
Celtic Sea	**69**		1	4		1		1		
England		**74**		1	2	4	8		3	
Isle of Man	1		**35**	2		2	2	7	2	1
Mid Wales	9		1	**54**	2	2	5		4	
N-Skerries		2		1	**27**	1	1	2		
N-Wales	1	10		5		**51**	2	2	1	1
Scotland		18		3	1	4	**83**	2	2	1
S-Skerries	5		9			1	6	**31**	1	
S-Wales	2	3	1	2		7	3	2	**54**	
SW-Ireland						1	2			**17**
Total No	76	92	52	77	34	73	114	53	74	20
No Correct	69	74	35	54	27	51	83	31	54	17
Proportion (%)	90.8	80.4	67.3	70.1	79.4	69.9	72.8	58.5	73.0	85.0

4.5.4. Classification of unknown juvenile trout parr to natal river.

In addition to establishing the microchemistry baseline for juvenile *Salmo trutta* from the 36 rivers in the Irish Sea region, the otolith chemistry of a further 39 fish was measured and the established baseline used to assign these fish to river of origin. This process was conducted "blind", *i.e.* with no prior knowledge of the actual river of origin of each fish until after the assignment process had been completed. For most of the fish, the probability of assignment to their most likely river of origin based on their otolith microchemistry was high (> 0.900). However, where assignment probability to the first choice river of origin was lower than P = 0.900, assignment probabilities to the second river of origin ranged from 0.109 to 0.460. Third choice river assignment probabilities, where present, ranged from 0.001 to 0.460 (see Table 4.15.). For 7 fish, the assignment technique did not extend beyond a single choice of river whilst for some fish, the probability of assignment to the first river of choice was very low and the likelihood of assignment to the second river of choice was almost as high (*e.g.* Fish 4, 27 and 32) and for some fish even the probability of the third choice river was high (*e.g.* Fish 26, 32)

In total 27/39 trout parr (*i.e.* 69 %) were correctly assigned to their river of origin (Table 4.15., Figure 4.13.) according to their Mg, Mn, Sr and Ba otolith chemistry. Where the assignment was successful, the probability of assignment was usually high with a mean assignment success of 0.926 ± 0.106 (range 0.540 – 1.000), however, probability was low (arbitrarily set at < 90%) for some fish (*e.g.* Fish IDs 4, 22, 33 and 39) (Table 4.15.). The classification results showed that for 6 trout parr (*i.e.* 15% of the data set), the correct assignment was for their 2nd choice of river (*i.e.* 15 %) with a predicted group probability ranging from 0.229 – 0.388. 5 trout parr (5/39, *i.e.* 12 %), which were misclassified to another river as their 1st choice, had a group probability ranging between 0.324 – 0.999 (Table 4.15.). These fish were found to be assigned to a river that was geographically distant from their actual river of origin (see Figure 4.13.). However, the predicted group classification for the remaining fish originally from the river Annan (Fish ID 3) was found to be assigned to an adjacent river (River Nith; see Table 4.15.).

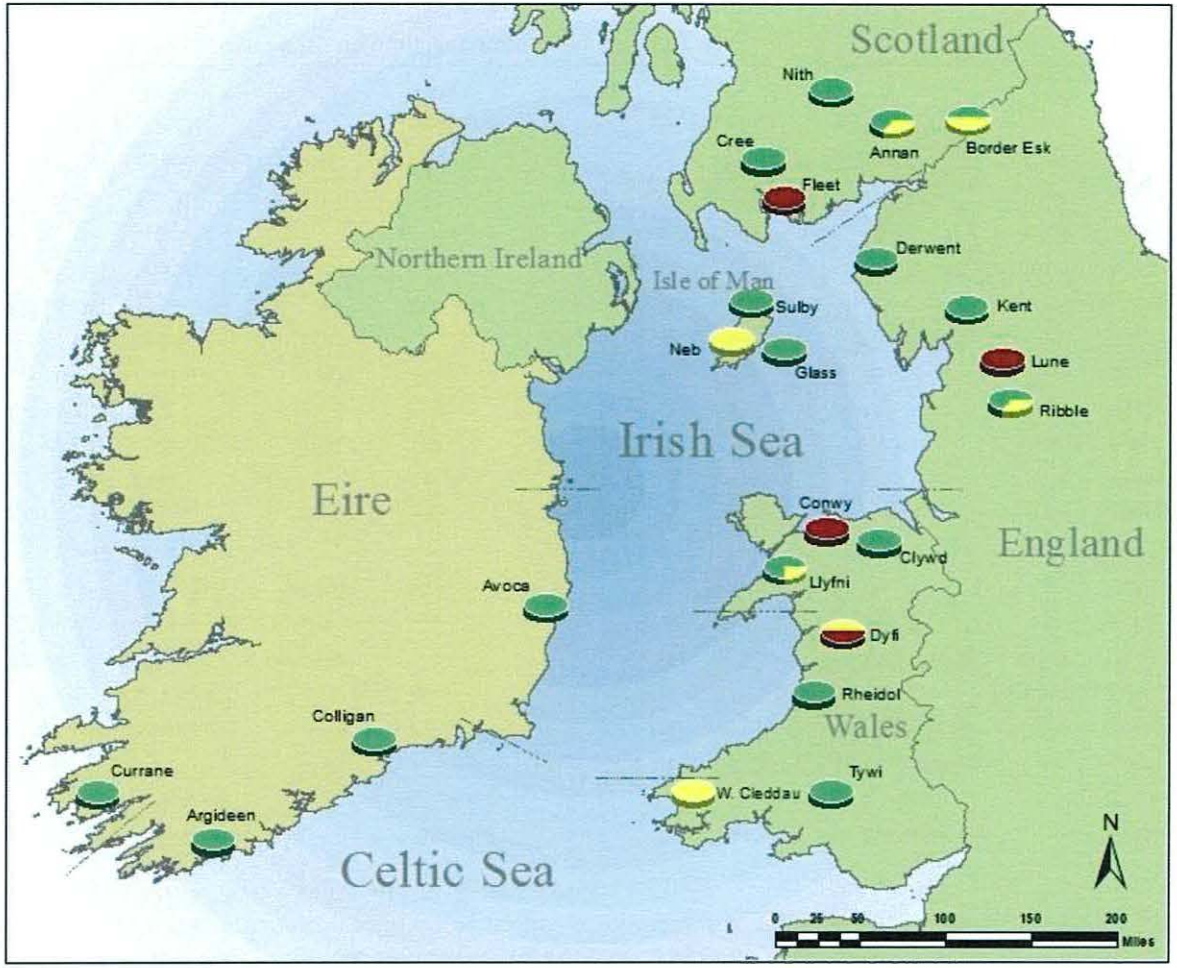


Figure 4.13. Map of the Irish and Celtic Sea region indicating the classification of the "unknown" *S. trutta* parr to their rivers of origin using cross-validation QDFA from Mg, Mn, Sr and Ba. Pie chart: **Green**- correct classification; **yellow** – 2nd choice; **red** – misclassified river.

Table 4. 15. Classification of juvenile *Salmo trutta* parr to their river of origin by means of the biogeochemistry baseline established using Mg, Mn, Sr and Ba obtained from the 36 rivers. 39 fish were assigned "blind" (*i.e.* no prior knowledge of origin) based on the probability of belonging to a particular river. Fish highlighted in **green** classify back to their correct river of origin (1st choice), fish highlight in **yellow** classify based on their 2nd choice assignment, with fish highlighted in **red** misclassified to a geographically distant river.

Fish ID	Actual Origin	Predicted 1st choice		Predicted 2nd choice		Predicted 3rd choice		Fish ID	Actual Origin	Predicted 1st choice		Predicted 2nd choice		Predicted 3rd choice	
1	**Llyfni**	**Llyfni**	$P = 0.999$	-----		-----		**21**	**Glass**	**Glass**	$P = 1.000$	-----		-----	
2	**Colligan**	**Colligan**	$P = 0.887$	Mawddach	$P = 0.113$	-----		**22**	**Derwent**	**Derwent**	$P = 0.779$	Tawe	$P = 0.192$	Dee (WR)	$P = 0.016$
3	Annan*	Nith	$P = 0.999$	-----		-----		23	Conwy	Castletown	$P = 0.854$	Kent	$P = 0.123$	Ehen	$P = 0.012$
4	**Currane**	**Currane**	$P = 0.540$	Annan	$P = 0.460$	-----		**24**	**Llyfni**	**Llyfni**	$P = 0.831$	Avoca	$P = 0.109$	Luce	$P = 0.050$
5	**Cree**	**Cree**	$P = 0.999$	Loughor	$P = 0.001$	-----		**25**	**Argideen**	**Argideen**	$P = 0.995$	Bandon	$P = 0.005$	-----	
6	Conwy	Derwent	$P = 0.999$	Mawddach	$P = 0.001$	-----		26	Lune	Dargle	$P = 0.464$	Castletown	$P = 0.224$	Dyfi	$P = 0.207$
7	**Nith**	**Nith**	$P = 1.000$	-----		-----		27	Dyfi	Lune	$P = 0.266$	Dyfi	$P = 0.229$	Dargle	$P = 0.171$
8	**Glass**	**Glass**	$P = 0.999$	-----		-----		28	Dyfi	Dargle	$P = 0.994$	Mawddach	$P = 0.006$	-----	
9	**Ribble**	**Ribble**	$P = 0.958$	Ehen	$P = 0.041$	Conwy	$P = 0.001$	29	Border Esk	Dee (WR)	$P = 0.736$	Border Esk	$P = 0.247$	Castletown	$P = 0.006$
10	**Annan**	**Annan**	$P = 0.995$	Currane	$P = 0.005$	-----		30	Neb	Dargle	$P = 0.607$	Neb	$P = 0.388$	Avoca	$P = 0.003$
11	**Tywi**	**Tywi**	$P = 1.000$	-----		-----		31	W. Cleddau	Mawddach	$P = 0.691$	W. Cleddau	$P = 0.296$	Clwyd	$P = 0.010$
12	**Ribble**	**Ribble**	$P = 0.984$	Conwy	$P = 0.007$	Ehen	$P = 0.007$	32	Fleet	Dargle	$P = 0.324$	Dyfi	$P = 0.287$	Castletown	$P = 0.260$
13	Ribble	Conwy	$P = 0.729$	Ribble	$P = 0.257$	Dee	$P = 0.004$	**33**	**Derwent**	**Derwent**	$P = 0.792$	Border Esk	$P = 0.208$	-----	
14	**Border Esk**	**Border Esk**	$P = 0.933$	Ehen	$P = 0.044$	Derwent	$P = 0.021$	34	Llyfni	Avoca	$P = 0.465$	Llyfni	$P = 0.362$	Loughor	$P = 0.102$
15	**Clwyd**	**Clwyd**	$P = 0.858$	W. Cleddau	$P = 0.138$	Teifi	$P = 0.004$	**35**	**Avoca**	**Avoca**	$P = 0.926$	W. Cleddau	$P = 0.024$	Glass	$P = 0.014$
16	**Rheidol**	**Rheidol**	$P = 1.000$	-----		-----		**36**	**Sulby**	**Sulby**	$P = 0.954$	Loughor	$P = 0.015$	Currane	$P = 0.011$
17	**Kent**	**Kent**	$P = 0.979$	Loughor	$P = 0.021$	-----		**37**	**Kent**	**Kent**	$P = 0.944$	Loughor	$P = 0.027$	Castletown	$P = 0.025$
18	**Annan**	**Annan**	$P = 0.999$	Currane	$P = 0.001$	-----		**38**	**Cree**	**Cree**	$P = 0.999$	Loughor	$P = 0.001$	-----	
19	**Sulby**	**Sulby**	$P = 0.946$	Currane	$P = 0.037$	Castletown	$P = 0.012$	**39**	**Clwyd**	**Clwyd**	$P = 0.785$	W. Cleddau	$P = 0.148$	Teifi	$P = 0.042$
20	**Llyfni**	**Llyfni**	$P = 0.915$	Avoca	$P = 0.073$	Sulby	$P = 0.005$								

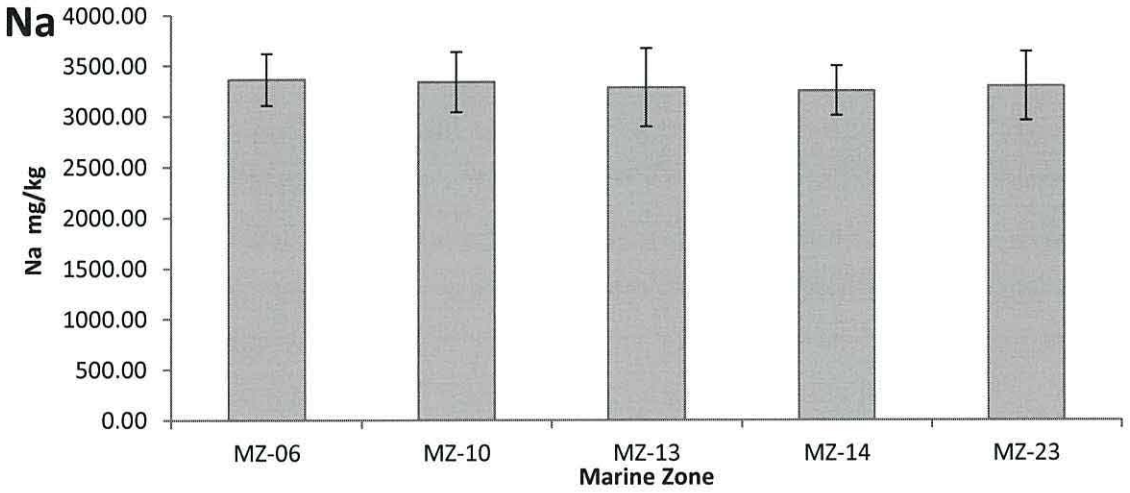
* NB. Annan assigned to Nith (adjacent river) with no other predicted choice.

4.6 Results of adult sea trout analyses

4.6.1. Analyzing the marine growth phase in adult sea trout otoliths

The element concentrations (expressed as mg/kg) in the section of the sagittal otolith transect corresponding to the marine phase of the lifecycle (determined from viewing the strontium profile, see Figure 4.4.) for the adult sea trout caught in the five different marine zones are presented in Figure 4.14. and Table 4.16. The data for Na indicated little variation in the elemental concentrations between the five marine zones (See Figure 4.14. and Table 4.16.). The element Mg indicated a higher elemental concentrations for marine zone 06 compared to the remaining 4 marine zones. However, for some elements, Figure 4.14. indicated increased variability in concentrations between one or more of the marine zone. Elemental concentrations observed between marine zones (MZ) indicated a 1.2 fold difference in the concentrations of K (381.742 – 457.299 mg/kg), and a 1.3 fold difference in the elemental concentrations of Mg (24.821 – 33.215 mg/kg) between marine zones. Similarly, Sr and Sn indicated 1.3 fold differences in concentrations between the five marine zones (1519.820 – 2038.200 mg/kg and 0.760 – 0.982 mg/kg respectively). The greatest difference in concentration between marine zones was observed for Mn which indicated a 2.3 fold difference between the five marine zones (1.646 – 3.855 mg/kg) most notably between MZ-13 and MZ-14 (see Table 4.16. and Figure 4.14.).

Significant differences were observed in the elemental concentrations of the Log_{10} transformed data for Na, Mg, K, Zn, and Ba (which exhibited normality and homoscedasity) in the marine phase of the adult sea trout otoliths between the five marine zones (MANOVA: using Wilks' criterion: $F_{20, 730} = 3.922$, $P < 0.001$). Individual ANOVA's conducted separately for each of the five elements (*i.e.* Na, Mg, K, Zn, and Ba) indicated highly significant differences between marine zones in their elemental concentrations for Mg ($F_{4, 228} = 8.26$, $P < 0.001$) and Ba ($F_{4, 228} = 5.36$, $P < 0.001$). Conversely, no significant differences were observed in the elemental concentrations of Na ($F_{4, 228} = 1.08$, $P = 0.366$) and Zn ($F_{4, 228} = 0.51$, $P = 0.726$) between marine zones, with K indicating a marginally significant difference in element concentration ($F_{4, 228} = 2.43$, $P = 0.048$) between each of the five zones.


Na
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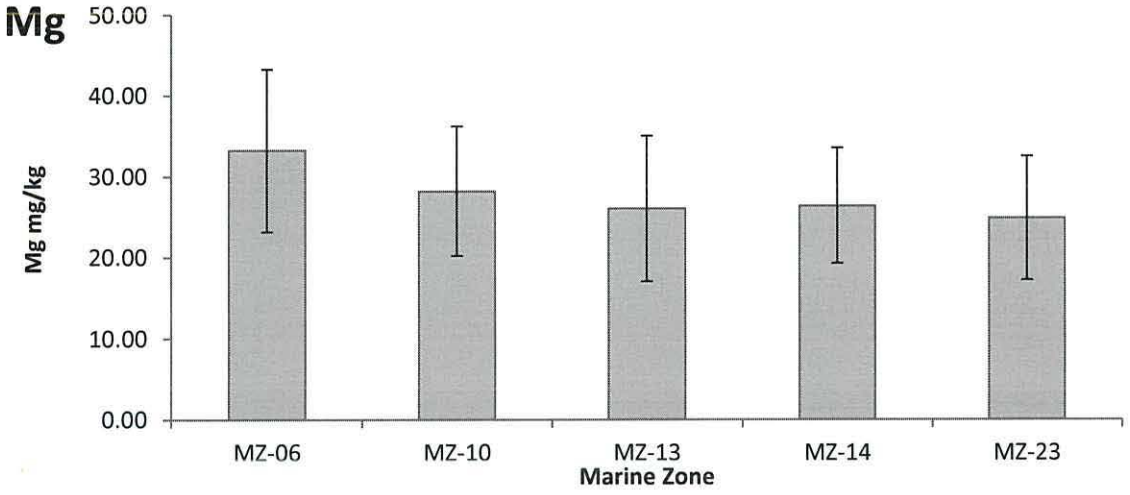
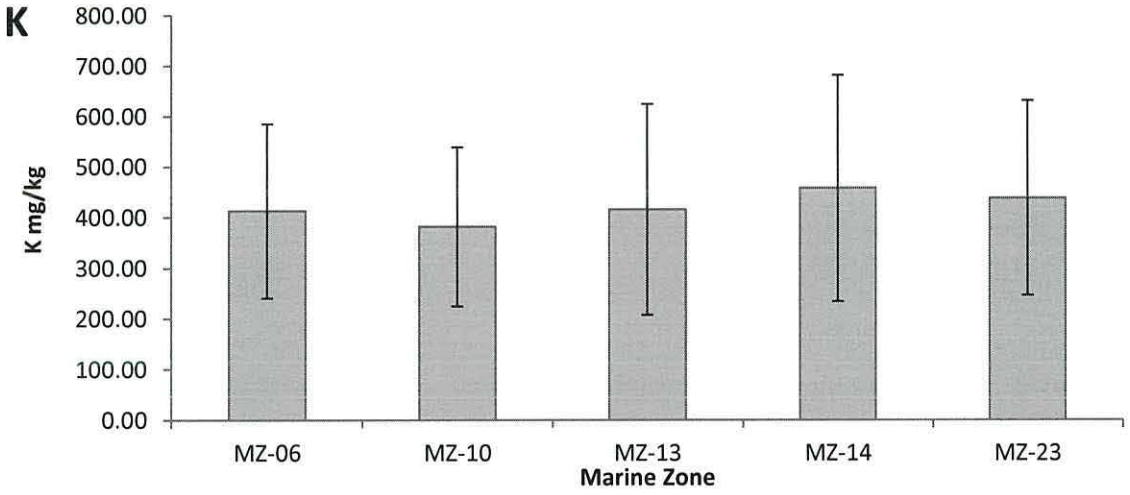
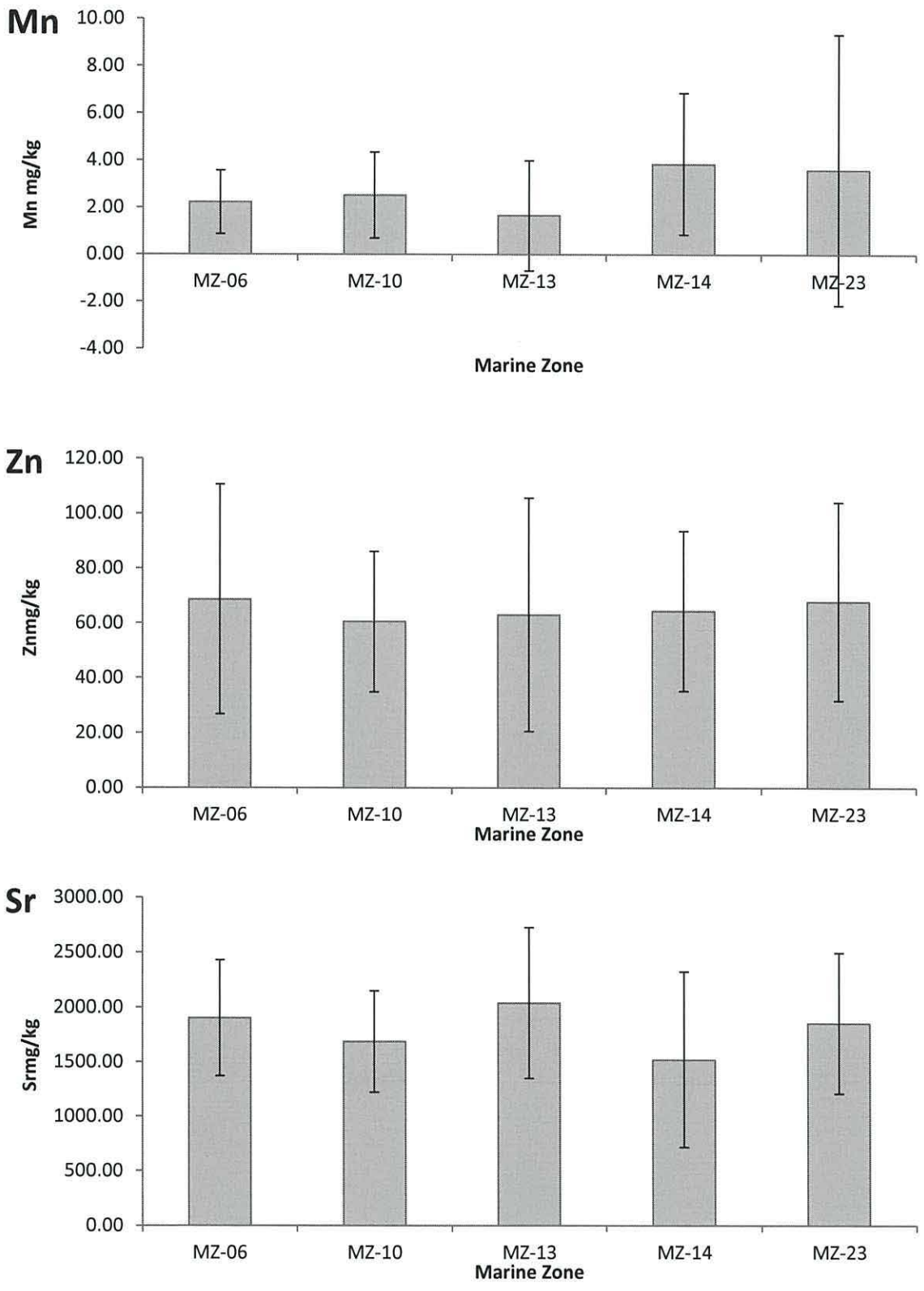
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
Na mg/kg
MZ-06
MZ-10
MZ-13
MZ-14
MZ-23
Marine Zone


Mg
50.00
40.00
30.00
20.00
10.00
0.00
Mg mg/kg
MZ-06
MZ-10
MZ-13
MZ-14
MZ-23
Marine Zone


K
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
K mg/kg
MZ-06
MZ-10
MZ-13
MZ-14
MZ-23
Marine Zone

(Figure 4.14. and caption are continued overleaf)



(Figure 4.14. and caption are continued overleaf)

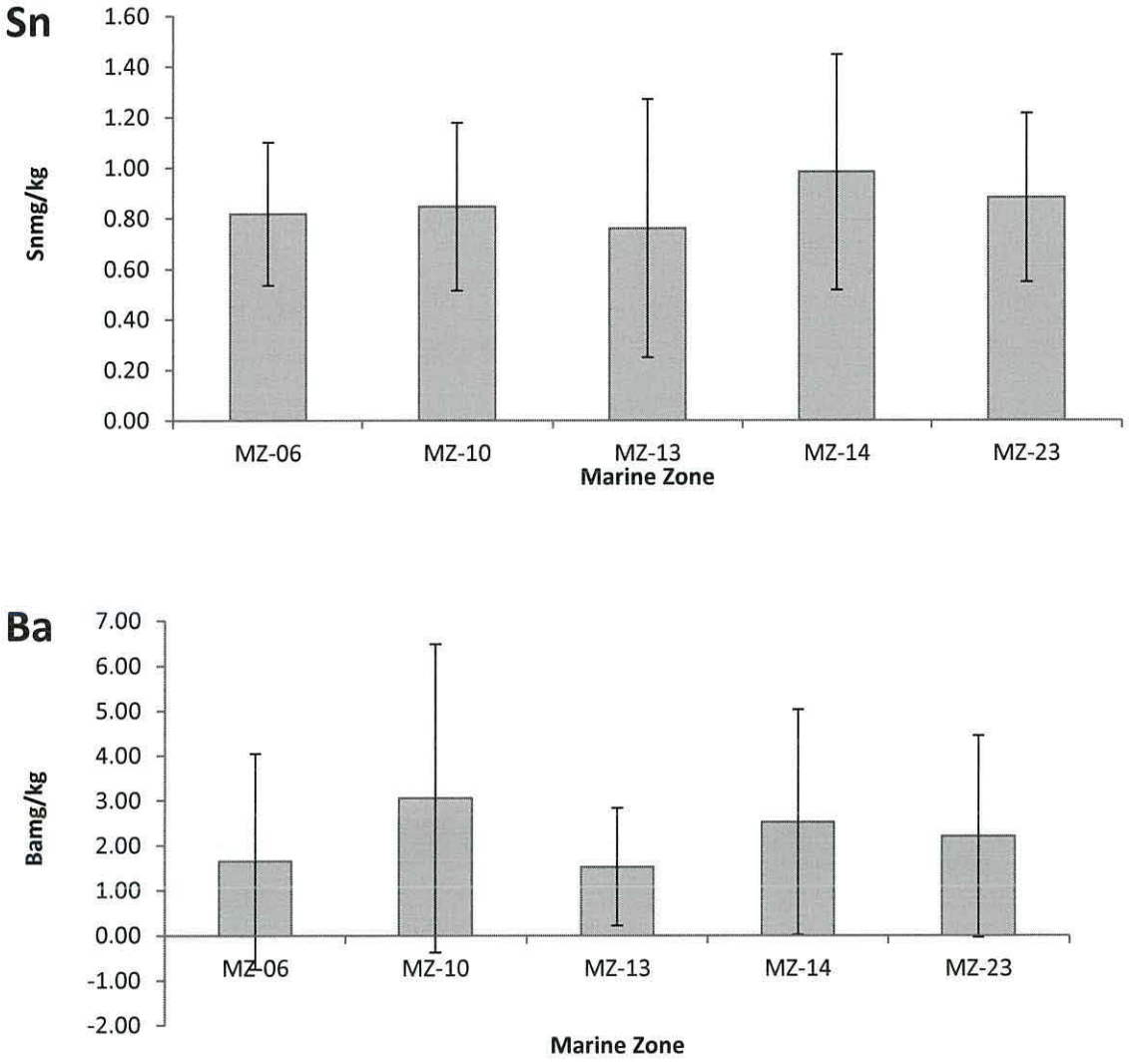


Figure 4.14. Mean marine phase concentrations (mg/kg) of the eight elements Na, Mg, K, Mn, Zn, Sr, Sn and Ba in adult sea trout otoliths for each of the five marine zones (MZ) of capture. Error bars are shown as ±1 standard deviation.

Table 4.16. Element concentrations (mg/kg) for the eight elements identified in the section of the otolith corresponding to the marine period of residency in marine-caught adult sea trout *Salmo trutta* caught in the five marine zones within the Irish Sea. Data are presented as mean ± 1 standard deviation. See Figure 4.3. for the location of each Marine Zone.

	Na	Mg	K	Mn	Zn	Sr	Sn	Ba	Total N°
MZ-06	3361.556 ±256.910	33.215 ±10.078	412.848 ±172.036	2.215 ±1.348	68.624 ±41.904	1899.289 ±529.692	0.817 ±0.284	1.648 ±2.400	66
MZ-10	3334.598 ±298.375	28.175 ±8.011	381.742 ±157.086	2.521 ±1.823	60.477 ±25.672	1684.167 ±463.421	0.846 ±0.332	3.058 ±3.428	71
MZ-13	3281.389 ±387.969	26.022 ±8.993	415.440 ±208.770	1.646 ±2.344	63.053 ±42.488	2038.200 ±687.503	0.760 ±0.510	1.525 ±1.311	24
MZ-14	3247.958 ±244.699	26.342 ±7.127	457.299 ±224.054	3.855 ±3.011	64.370 ±29.195	1519.820 ±803.328	0.982 ±0.465	2.519 ±2.506	32
MZ-23	3292.977 ±340.827	24.821 ±7.651	438.124 ±192.226	3.595 ±5.749	67.873 ±36.172	1852.152 ±643.484	0.881 ±0.333	2.204 ±2.239	36

Bonferroni *post-hoc* pairwise comparisons indicated that otolith concentrations of Mg in the marine growth phase of the otoliths taken from sea trout captured in MZ-06 were significantly higher than the concentrations observed in the otoliths from the other four marine zones (all $P < 0.01$). Similarly, otolith concentrations of Ba for sea trout captured in MZ-10 were significantly higher than those captured in MZ-06 ($P < 0.001$) and MZ-13 ($P < 0.031$). *Post-hoc* pairwise comparisons (using a Bonferroni test) indicated no significant differences in the elemental concentrations of K between each the five marine zones.

Assessments of the elements failing the assumptions for parametric analysis (*i.e.* Mn, Sr and Sn; Levene's $P < 0.05$) were conducted using the non-parametric Kruskal-Wallis test. Results indicated no significant differences in the elemental concentrations of Sr and Sn between the five marine zones (Sr: $K_{4,228} = 9.42$, $P = 0.051$; Sn: $K_{4,228} = 3.08$, $P = 0.554$ respectively). However, although the element Mn indicated significant differences in concentrations between the five marine zones ($K_{4,228} = 10.67$, $P = 0.030$), pairwise comparisons using a Mann-Whitney U test indicated no significant differences between marine zones (all $P > 0.05$ see Table 4.17.).

Table 4.17. Results conducted on the adult trout otoliths using the Non-parametric Mann-Whitney test to assess the Log_{10} element Mn concentrations between each of the five marine zones 06, 10, 13, 14 and 23.

					95% C.I.		
Marine Zone	**Zone**	**n**	**Median**	**W**	**Lower**	**Upper**	***P***
MZ-06		**64**	-2.7072				
	MZ-10	**69**	-2.7017	4219.0	-0.0614	0.0465	0.7577
	MZ-13	**23**	-2.5935	2692.5	-0.1832	0.0559	0.2365
	MZ-14	**30**	-2.6869	2872.5	-0.1250	0.0204	0.1756
	MZ-23	**34**	-2.7114	3074.0	-0.0963	0.0476	0.4853
MZ-10		**69**	-2.7017				
	MZ-13	**23**	-2.5935	3098.0	-0.1703	0.0687	0.3213
	MZ-14	**30**	-2.6869	3293.5	-0.1256	0.0288	0.2349
	MZ-23	**34**	-2.7114	3509.5	-0.0934	0.0531	0.5844
MZ-13		**23**	-2.5935				
	MZ-14	**30**	-2.6869	619.5	-0.1383	0.1456	0.9857
	MZ-23	**34**	-2.7114	702.0	-0.0915	0.1586	0.5747
MZ-14		**30**	-2.6869				
	MZ-23	**34**	-2.7114	1031.0	-0.0684	0.1323	0.4553

Using quadratic discriminant function analysis (QDFA) with the elements that were normally distributed, but with / without equal variance (*i.e.* Na, Mg, K, Mn, Zn, Sn and Ba) and with marine zone set as the variable and the elements set as the predictors, classification of adult sea trout back to their region of capture was low with only 114/219 adults (52%) correctly classified back to their marine zone of capture (Table 4.18.). CV-QDFA classification accuracy was reduced further with 72/219 (33%) marine-caught sea trout correctly classifying back to their marine zone of capture (see Table 4.19.). Cohen's kappa statistics indicated that the chance-corrected classification accuracy of trout parr to marine zone of capture using QDFA was 0.45 (C.I. 0.36 – 0.54) but was very low when using the leave-one-out CV-QDFA approach reducing to 0.14 (C.I. 0.05 – 0.21) which suggests that some correct classifications are chance-associated.

Using principal component analysis (PCA) the standard correlation matrix indicated Sn and Mn were the most important elements explaining the variation and discrimination between the five marine zones sampled for adult trout, with component scores of 0.527 and 0.147 respectively. Principal component 1 indicated a variance (eigenvalue) of 2.050 and accounted for 29% of the total variance, with PC 2 indicated an eigenvalue of 1.695 and explaining 24% of the variance observed (see Figure 4.15.). Both principal components 1 and 2 showed a combined discriminating factor of 54%, with the first four principal components representing 81% of the combined total variability observed for the five marine zones using the otolith trace elements Na, Mg, K, Mn, Zn, Sn and Ba. The PCA plot (Figure 4.15.) indicated no discernible zonal grouping between each of the five marine zones for the sea trout using the elemental concentrations of Na, Mg, K, Mn, Zn, Sn and Ba.

Table 4.18. QDFA using the Log_{10} elements Na, Mg, K, Mn, Zn, Sn and Ba to predict marine zone of capture of adult sea trout (*Salmo trutta*) using material ablated from the marine phase of the otoliths. Correct number of adults to sub-region highlighted in bold.

	Marine Zone 06	Marine Zone 10	Marine Zone 13	Marine Zone 14	Marine Zone 23
Marine Zone 06	**32**	14	1	5	1
Marine Zone 10	13	**36**	1	3	8
Marine Zone 13	8	6	**14**	3	6
Marine Zone 14	9	9	3	**17**	4
Marine Zone 23	2	4	3	2	**15**
N°	64	69	22	30	34
N° correct	32	36	14	17	15
Proportion	0.500	0.522	0.636	0.567	0.441

Table 4.19. CV-QDFA using the Log_{10} elements Na, Mg, K, Mn, Zn, Sn and Ba to predict marine zone of capture of adult sea trout (*Salmo trutta*) using material ablated from the marine phase of the otoliths. Correct number of adults to sub-region highlighted in bold.

	Marine Zone 06	Marine Zone 10	Marine Zone 13	Marine Zone 14	Marine Zone 23
Marine Zone 06	**27**	16	4	6	4
Marine Zone 10	17	**28**	1	5	9
Marine Zone 13	9	8	**7**	5	10
Marine Zone 14	9	11	4	**6**	7
Marine Zone 23	2	6	6	8	**4**
N°	64	69	22	30	34
N° correct	27	28	7	6	4
Proportion	0.422	0.406	0.318	0.200	0.118

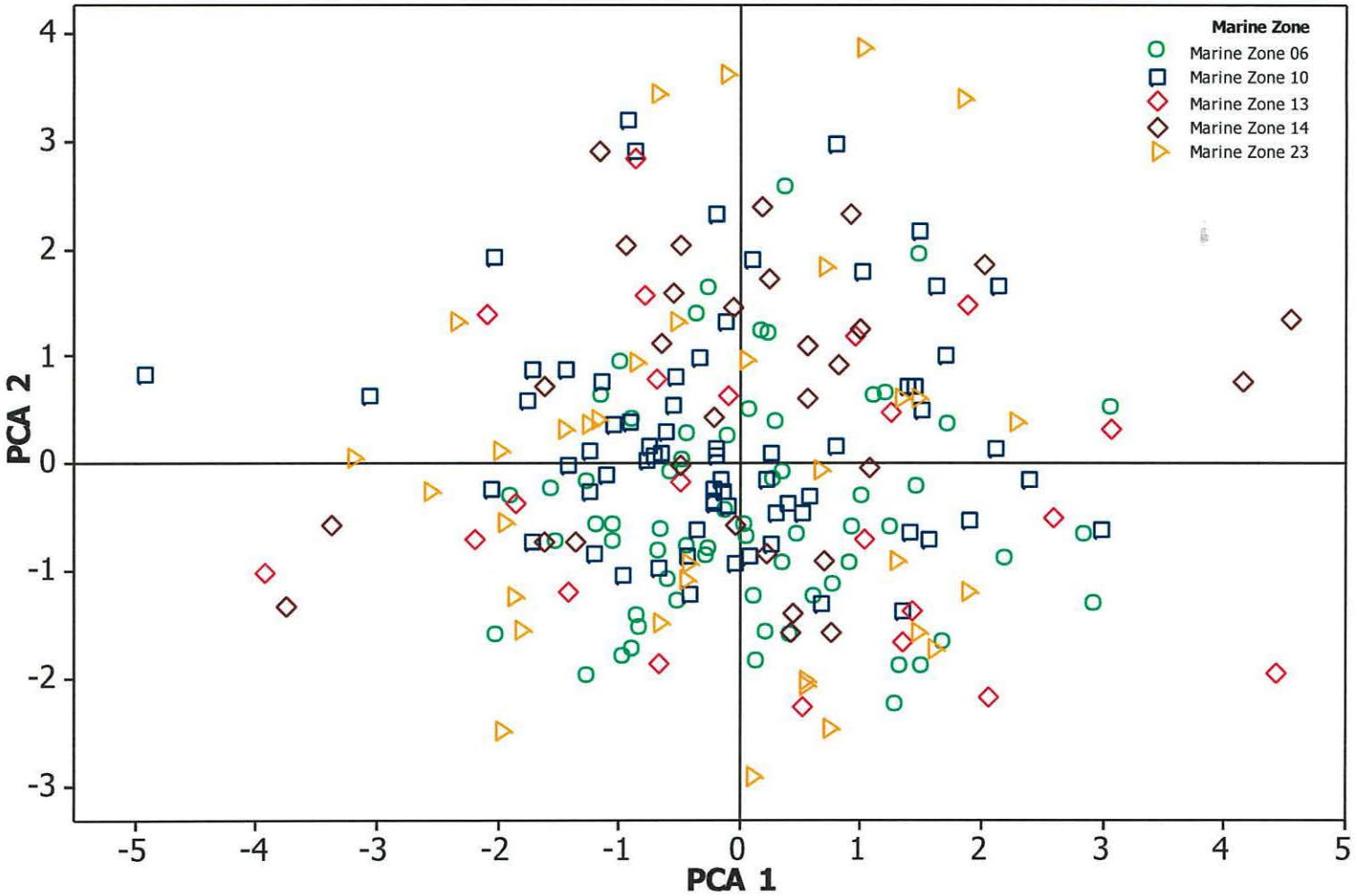


Figure 4.15. PCA plots using the Log_{10} elements Na, Mg, K, Mn, Zn, Sn and Ba to predict marine zone of capture of adult sea trout (*Salmo trutta*) using material ablated from the marine phase of the otoliths.

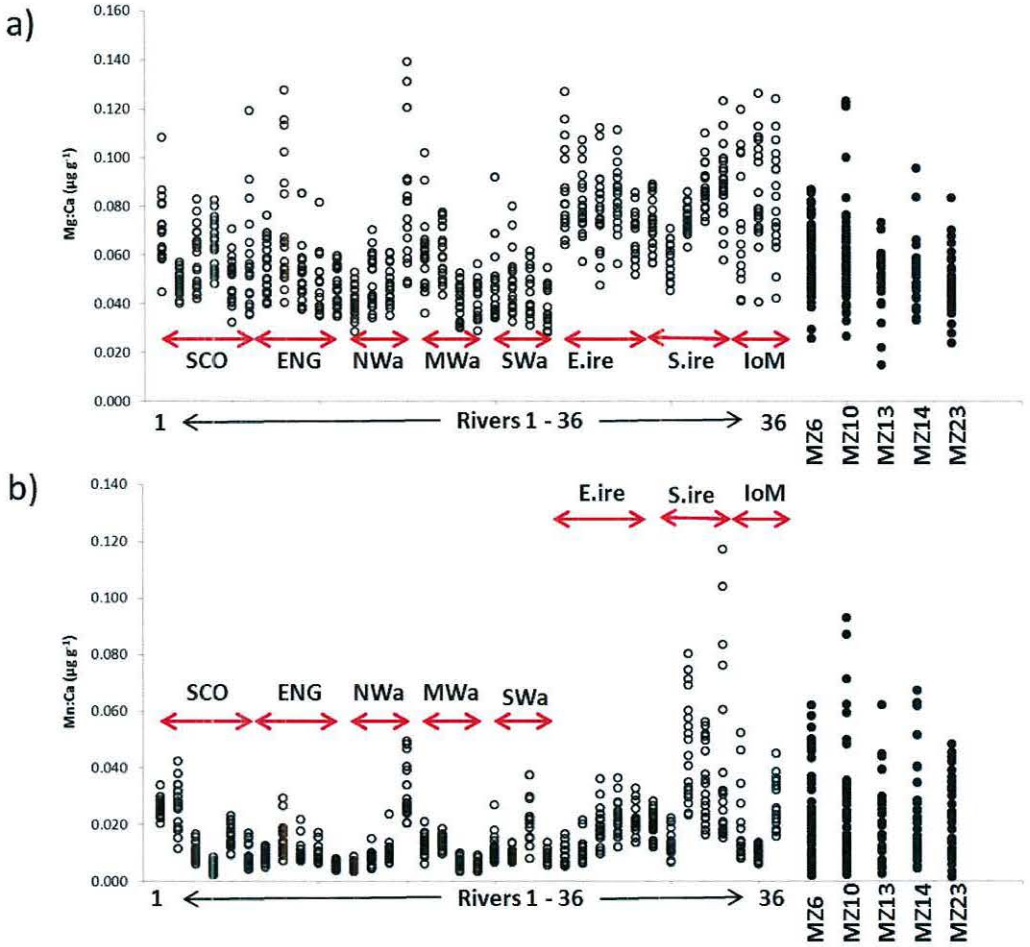
4.6.2. Analysis of freshwater growth in adult sea trout otoliths and assignment to region

Mean element:Ca ratio concentrations calculated for the part of the adult sea trout sagittal otolith corresponding to the juvenile parr phase (see Figure 4.4. for identification of the freshwater growth period using Sr profile) are presented alongside the concentrations for the juvenile parr baseline data for the 36 rivers in Figure 4.16. and Table 4.20. If adult sea trout remain in coastal waters close to their natal rivers to feed prior to returning to spawn than it would be expected that the chemistry for the juvenile parr of their otolith would be similar to the otolith chemistry of the parr from the rivers in the adjacent coastal region. However, there was poor correspondence between the elemental concentrations in the otoliths of the freshwater-resident parr and in the freshwater water sections of the adult sea trout otoliths. Figure 4.16. shows that for each of the elements there is considerable scatter in the freshwater chemistry for the adult sea trout with their range of parr chemistry values overlapping with much of the freshwater baseline data. There was no correlation between the average element:Ca ratios for Mg, Mn and Sr in central (parr) section of the otoliths from the adult sea trout caught in each marine zone and the average freshwater value for the adjacent coastal region, however, a significant correlation was reported for Ba ($r = 0.987$, $P = 0.002$).

Principal component analysis (PCA) of the elemental concentrations of Mg, Mn, Sr and Ba ablated from the juvenile portion of marine-caught sea trout otoliths indicated that Mn (0.653) and Ba (0.087) were the most important elements in explaining the variation and discrimination observed between the five marine zones and the ten sub-regions (Figure 4.17.). Principal component 1 (PC1) indicated a variance (eigenvalue) of 1.639 explaining 41% of variance observed whilst PC2 indicated an eigenvalue of 1.037 and explained 26% of the variance observed (see Figure 4.17.). Together PC 1 and 2 explained 67% of the variance and the first three principal components represented 88% of the combined total variability observed. However, the PCA plot (Figure 4.17.) indicated no distinct zonal clustering marine zones and freshwater sub-regions based on Mg, Mn, Sr and Ba otolith chemistry.

Table 4.20. Element concentrations (mg/kg) for the four elements identified in juvenile trout parr otoliths from 10 sub-regions and the section of the otolith corresponding to the freshwater period of residency in marine-caught adult sea trout *Salmo trutta* captured in the five marine zones within the Irish Sea. Data are presented as mean ± 1 standard deviation. See Figure 4.3. for the location of each Marine Zone.

	Mg	Mn	Sr	Ba	Total N°
S-W Scotland	2.968 ± 0.797	0.646 ± 0.392	72.430 ± 27.748	1.432 ± 0.835	114
SW-Scotland (MZ10)	2.333 ± 0.729	0.808 ± 0.742	51.344 ± 21.123	1.398 ± 881	73
N-W England	2.672 ± 0.875	0.467 ± 0.228	40.845 ± 10.142	0.987 ± 0.330	92
North Wales	2.613 ± 1.043	0.659 ± 0.567	57.116 ± 19.828	0.537 ± 0.223	73
N-Wales (MZ13)	2.036 ± 0.521	0.815 ± 0.585	47.310 ± 22.722	0.481 ± 0.262	26
N-Wales (MZ14)	2.093 ± 0.499	0.914 ± 0.674	45.217 ± 15.436	0.548 ± 0.353	34
Mid Wales	2.481 ± 0.734	0.472 ± 0.221	49.332 ± 10.494	0.363 ± 0.247	77
South Wales	2.176 ± 0.639	0.596 ± 0.341	42.191 ± 19.778	1.133 ± 0.745	74
North Skerries	4.007 ± 0.803	0.477 ± 0.195	37.079 ± 13.219	0.842 ± 0.149	34
South Skerries	3.811 ± 0.808	1.014 ± 0.300	67.832 ± 8.511	0.699 ± 0.396	53
S-Skerries (MZ06)	2.277 ± 0.524	0.785 ± 0.603	49.817 ± 16.165	0.794 ± 0.656	69
Celtic Sea	3.141 ± 0.691	0.803 ± 0.276	41.219 ± 8.734	0.293 ± 0.115	76
S-W Eire	4.323 ± 0.851	1.918 ± 1.560	151.542 ± 44.201	2.067 ± 2.462	20
Isle of Man	3.887 ± 1.157	0.905 ± 0.564	71.662 ± 14.034	0.654 ± 0.250	52
Isle of Man (MZ23)	1.896 ± 0.476	0.854 ± 0.529	71.192 ± 22.933	0.714 ± 0.381	37



(Figure 4.16. and caption are continued overleaf)

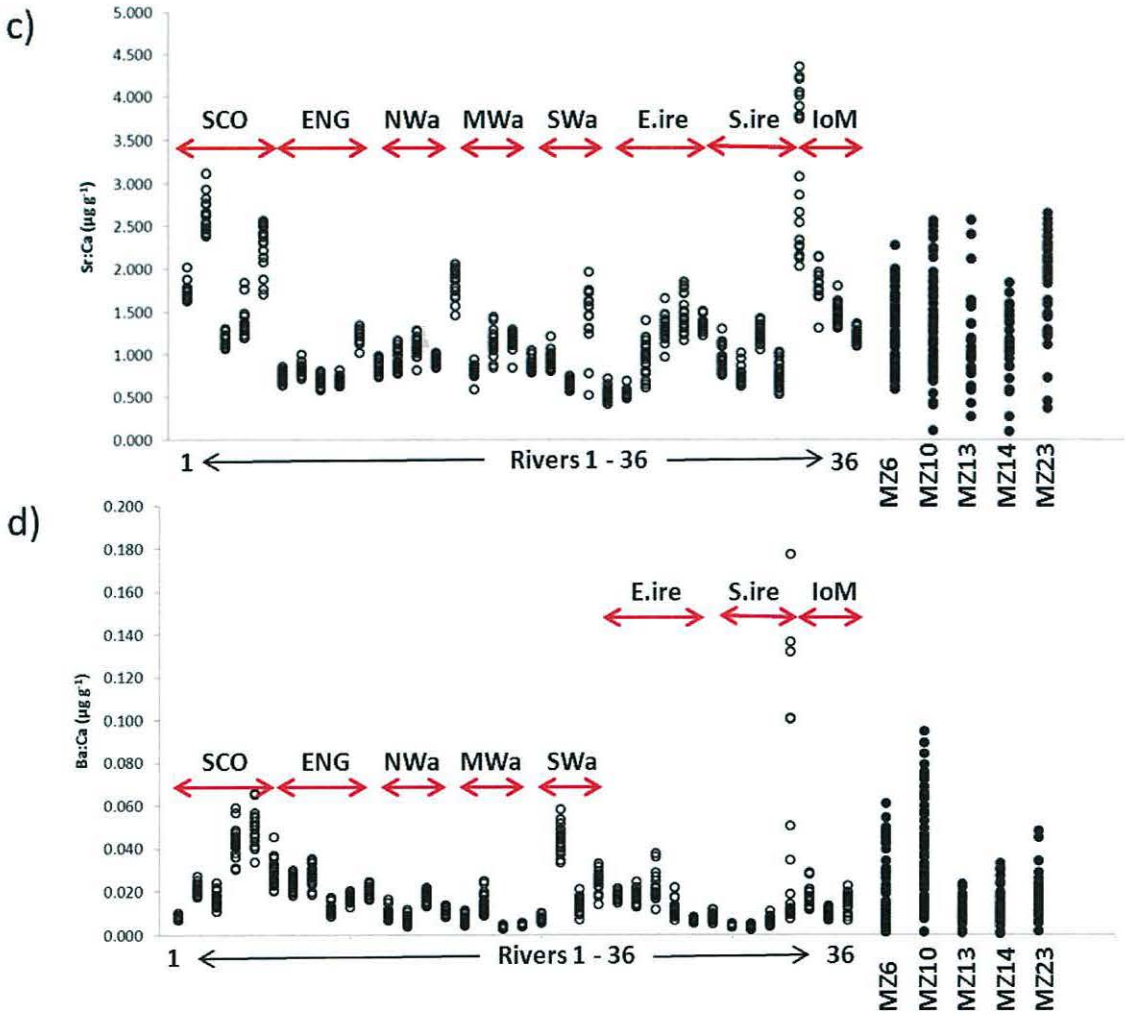


Figure 4.16. Mean element concentrations (expressed as element:Ca ratios) for a) Mg, b) Mn, c) Sr and d) Ba in the otoliths of parr from the 36 rivers sampled to establish the freshwater baseline (open circles; listed in order from 1-36, see Table 4.2. for river codes) and in the central section (parr phase) of adult sea trout otoliths (solid circles) from each of the five marine zones of capture.

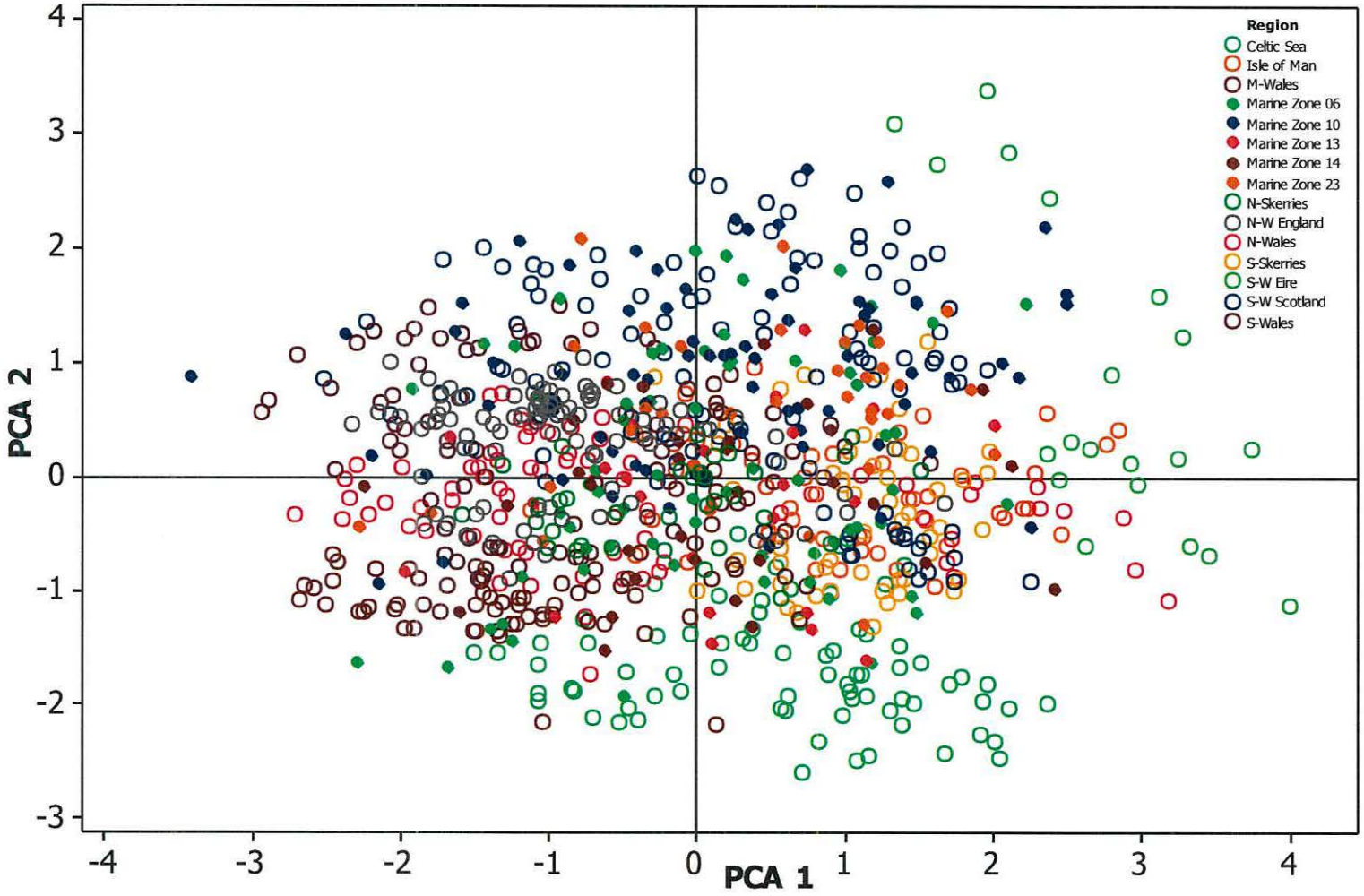


Figure 4.17. PCA plots using the Log_{10} elements Mg, Mn, Sr and Ba obtained from the parr baseline to predict region of origin for adult sea trout (*Salmo trutta*) using otolith material ablated from the freshwater growth period from adult trout captured from the five marine zones within each region.

Using the established juvenile trout parr microchemical baseline (Table 4.10.) created from juvenile parr sampled in the 10 sub-regions around the Irish Sea, each adult sea trout was assigned to a putative sub-region of origin based on the chemistry of the juvenile parr phase in the center of their otolith using QDFA. If adult sea trout remain in the coastal waters close to their natal river of origin to feed prior to returning to spawn then it would be expected that they would classify back to the coastal sub-region adjacent to their MZ of capture. In fact, classification back to adjacent coastal sub-region was low at 17.7% with 41/231 adult trout correctly assigned to adjacent coastal region based on their freshwater chemistry in the center of their otolith (see Table 4.21.). The chance-corrected QDFA classification accuracy to region for the adult sea trout using Cohen's kappa statistic was low at 0.15 (± C.I 0.048), indicating that many of the correct classifications recorded may have been a result of chance. However, it is interesting to note that a large number of the MZ23 fish (25/36, 69%) classified to the nearby SW Scotland region suggesting that *Salmo trutta* from the Solway Firth rivers may be utilizing the coastal waters of the Isle of Man as a feeding ground.

Table 4.21. QDFA Predicted Classification of adult marine caught sea trout *Salmo trutta* using the freshwater growth phase to their region of origin by means of the biogeochemistry baseline established using Mg, Mn, Sr and Ba obtained from the 36 rivers. Adult fish were assigned based on the probability of belonging to a particular region. Correct number of parr highlighted in **bold**.

	MZ-10	**MZ-13**	**MZ-14**	**MZ-06**	**MZ-23**
Region					
S-W Scotland	**25 (34.7%)**	3	3	14	25
N-W England	13	2	3	6	1
North Wales	3	**4 (16.6%)**	**1 (3.1%)**	8	3
Mid Wales	3		2	14	2
South Wales	14	4	8	8	1
North Skerries					
South Skerries	5		2	**3 (11.9%)**	
Celtic Sea		6	5	5	1
S-W Ireland	5	1	1	2	
Isle of Man	4	4	7	7	**3 (8.3%)**
Total N°	72	24	32	67	36
N° correct	25	4	1	8	3
Proportion	0.347	0.167	0.031	0.119	0.083

4.7. Discussion

Tracking and understanding the movement patterns of fishes and identifying individuals back to their natal origins or juvenile nursery grounds are central to understanding the ecology of a fish species and for the effective management of commercial species. The use of multi-elemental otolith microchemistry is becoming a useful tool for stock discrimination purposes and the reconstruction of individual migratory histories (Elsdon and Gillanders, 2003*a*). For example, this approach has enabled spatial geochemical differences and discrete populations within local areas to be identified and assisted in the reconstruction of the movement patterns and intermixing of fishes from these different populations (*e.g.* Elsdon and Gillanders, 2003*a*; Swearer *et al.*, 2003; Veinott and Porter, 2005; Elsdon *et al.*, 2008; Ramsay *et al.*, 2011; Tanner *et al.*, 2012; Veinott *et al.*, 2012).

4.7.1 *Spatial variability in biogeochemical tags*

The results of this study have shown that otolith element:Ca ratios of Mg, Mn, Sr and Ba varied significantly between trout parr from the 36 rivers. Moreover, a distinct geographical pattern was observed with elevated Mg:Ca and Mn:Ca ratios in the otoliths of parr sampled from Irish and Manx rivers draining into in the Irish Sea compared to those sampled from Western GB rivers (see Figure 4.5. and Table 4.6.). Similarly, Sr:Ca ratios showed elevated levels in the otoliths of trout parr sampled from the six rivers in southwest Scotland, the three rivers on the Isle of Man and the Currane in southwest Ireland compared to the other river samples (see Figure 4.5.). One river in particular, the Currane in southwest Ireland, exhibited a very distinctive chemical signature and showed elevated concentration levels of Mn:Ca, Sr:Ca and Ba:Ca ratios when compared to the remaining rivers. Taken together, these elemental differences indicate evidence of a possible trans-Irish Sea concentration gradient within the otolith chemistry of trout parr.

Element:Ca ratios observed in fish otoliths are thought to be largely determined by water chemistry (Veinott and Porter, 2005; Martin and Wuenscel, 2006), with studies by Wells *et al.* (2003*a*), Walther and Thorrold (2008) and Ramsay *et al.* (2011) indicating a correlation between ambient stream water chemistry and the concentrations of Mg, Mn, Sr and Ba in the calcified structures of fish (*e.g.* otoliths and scales). However, it is thought that otolith concentrations of some elements may also be influenced by dietary

intake (see Buckel *et al.*, 2004 and references therein). In addition, studies in the marine environment have also found a direct correlation between Sr:Ca and Ba:Ca ratios in seawater and otoliths (Bath *et al.*, 2000) and between Sr:Ca in the otoliths of fish and estuarine water (Kraus and Secor, 2004). It is the transfer of these unique geochemical signals from the water to the otolith that allows fish to be identified back to their natal origin based on a unique otolith fingerprint (*e.g.* Veinott and Porter, 2005; this study).

Within the geographic area covered in the present study, the geological bedrock within the river catchments sampled for *S. trutta* parr has been shown to vary (B.G.S, 2013). The west coast of Scotland comprises primarily of metamorphic bedrock compared to a mixture of Carboniferous and Triassic bedrock in northwest England and a mixture of both Carboniferous and Silurian bedrock in Wales (B.G.S, 2013). The bedrock for the rivers and streams sampled from the east coast and the south west coast of Ireland comprise mainly of Carboniferous, Devonian and Silurian geology (B.G.S, 2013). Within the study area, these differences in underlying geological bedrock that differ in their elemental composition, and in the rates at which the rocks erode and release elements into the surface sediments and stream water, are probably one of the drivers behind the elevated concentrations for Mg and Mn observed in Ireland and the Isle of Man compared to the west coast of the UK. An evaluation of the bedrock underlying these rivers and their water chemistry were beyond the scope of the present study. However, previous studies have identified spatial geological heterogeneity, together with land use, as the major drivers of differences in ambient water chemistry (B.G.S. 1999) and therefore the key factor in discriminating between fish using element: Ca ratios within otolith aragonite (see Campana, 1999; Vasconcelos *et al.*, 2007). Thus, in the present study spatial heterogeneity in element chemistry has presented a unique chemical fingerprint in trout parr otoliths with fish being correctly classified back to their river of origin with an accuracy in excess of 70% (and up to 100%, *e.g.* Nith, Annan, Tywi, Argideen and Currane) for 21/36 rivers studied (see Figure 4.7., Table 4.8.). This high classification accuracy is based on the assessment of element:Ca ratios for 4 elements in the otolith: Mg Mn, Sr and Ba. These are a small suite of trace metals which substitute for Ca and, in the case of Mn, Sr and Ba, have a similar ionic radii and ionic charge which matches the free Ca^{2+} cation within the aragonite matrix of otoliths (Swearer *et al.*, 2003; Hedges *et al.*, 2004; Clarke *et al.*, 2007). In addition Mg, which has been described by some authors as being resilient to reabsorption and is assumed to substitute for Ca within the lattice

structure of the otoliths (Rooker *et al.*, 2001; Swan *et al.*, 2006), is another trace metal which is important in discriminating between geographic locations and is routinely used as a biogeochemical tags in fish otolith microchemistry studies (*e.g.* Wells *et al.*, 2000, 2003*a*; Muhlfeld *et al.*, 2005; Ramsay *et al.*, 2011).

The ability to identify individual fish back to a specific water body will require a minimum residence time in order to for the unique chemical tag to be deposited in the otolith. Previous studies have indicated fish need to be resident in a particular location for at least one month (see Elsdon and Gillanders 2003*b*, 2005) to allow the chemistry of the local environment to be taken up within the calcified structures. However, residence time will also be dependent on the size of the otolith, its growth rate and the technique used to measure elemental concentrations since LA- or sb-ICP-MS will require different sample sizes of otolith material. Previous studies have worked with species where habitat residence times have been assumed to anything from a few months up to a few years dependent upon the species (see Beck *et al.*, 2001; Elsdon and Gillanders 2003*b*; Able, 2005; Vasconcelos *et al.*, 2008). In the present study *S trutta* parr were sampled before undergoing smoltification and although not aged during the present study, were assumed to have resided in their natal rivers for 1-2 years prior to capture.

4.7.2 *Classification to river based on otolith microchemistry*

In biogeochemical tagging studies divalent trace elements such Mg, Mn, Sr and Ba are often the most important elements identifying fish back to source using discrimination analysis (*e.g.* see Muhlfeld *et al.*, 2005; Veinott and Porter, 2005; Ramsay *et al.*, 2011). In the present study, the concentrations of these elements in the otoliths of *Salmo trutta* parr were found to be excellent natural tags in identifying the natal origin of fish back to the 36 rivers which drain into the Irish and Celtic Seas. Overall cross-validation classification accuracy was high with 74% of the trout parr correctly assigned back to their source river; for most rivers used in this study (*i.e.* 21/36) assignments success ranged between 70% - 100%. The use of the Cohen's Kappa statistic (see Barnett-Johnson *et al.*, 2008; Ramsay *et al.*, 2011) in the present study, with a calculated value of 0.73 ± 0.04 C.Is, provides confidence that the high level of correct assignment has not occurred as a result of chance.

The methodological approach adopted in this study - *i.e.* the collection of fish from a number of potentially geochemically distinct sources, the assessment of the trace element composition in the otoliths of these fish and the use of an assignment test (such as discriminant function analysis) to try to identify fish back to source – is a standard approach that has been adopted in many fish studies in the last decade (Table 4.22.). Studies using this approach have shown that classification accuracies of between 70 % and 100% *i.e.* similar to that achieved in the present study are not unusual (*e.g.* Thorrold *et al.*, 1998*a*; Gillanders and Kingsford, 2000; de Pontual *et al.*, 2000; Gillanders and Kingsford, 2003*b*; Dorval *et al.*, 2005; Vasconcelos *et al.*, 2007).

Although many studies show classification successes ranging between 70 and 100% it is important to note that these studies have differed in their spatial coverage and the number of source populations being studied. To the author's knowledge, the present study includes one of the largest number of source populations (*i.e.* 36) and covers one of the largest geographical areas (2,400 km). Spatial variation in otolith microchemistry and its use as a biogeochemical tag has been reported in numerous earlier studies: see Gillanders *et al.* (2001) for an extensive summary list and Elsdon *et al.* (2008) and Table 4.22. for a more recent review. However, most studies have sampled from sites distributed over relatively small spatial scale, for example from sites separated by a few hundred metres (*e.g.* Gillanders and Kingsford, 2000) or by < 1 km to locations separated by < 10 km (see Table 4.22.). It is interesting to note that sampling sites separated by as little as a few metres can be distinguished based on discrete otolith elemental signatures (Gillanders and Kingsford, 2000) and fish from these sites can be classified with a high degree of accuracy (60% - 100%), highlighting the sensitivity of this technique. Regional differences in otolith microchemistry signatures within freshwater catchments have suggested that it might be possible to identify fine-scale movement patterns of fishes, as suggested by Ramsay *et al.* (2011), based on work conducted in the River Dee (a small upland river catchment) on 6 sampling sites (approximately 7.5 km separating neighbouring sampling sites). The combination of otolith trace element and strontium isotope chemistry may present a powerful technique in examining the in-river migrations of fishes (see Walther and Thorrold, 2008; Walther *et al.*, 2008). As expected, with an increase in spatial scale (*i.e.* > 10 km between sampling sites), discrete, distinct trace elemental signatures can be identified (see Table 4.22.).

Table 4.22. Summary of recently published data examining spatial variability in otolith chemistry over a range of geographical scales and its potential to infer movement patterns of fish. Data are organised by year of reference. Freshwater studies are shaded blue.

Study Location	Sample design	Geographical Range	Species	Sampling Year/s	Sample N°	Chemicals Analysed	Differences	Classification success	Author/s
East coast New South Wales, Australia	7 estuaries 2-5 sites/estuary	600 km	*Pelates sexlineatus*	1998-1999	10 / site	Mn, Sr, Ba	Difference within and amongst estuaries	Within & among estuaries 60 – 100 %	Gillanders & Kingsford, 2000
South-eastern Australia	6 Inlets Between 1-9 sites / inlet	300 km	*Pagrus auratus*	2000-2001	Range 2-56 / inlet	Mn, Sr, Ba	Difference between Inlets & year classes	Between inlets 85-98 %	Hammer *et al.*, 2003
Idaho USA	3 streams	60 km	*Oncorhyncus clorki lewisi*	2000	10 / stream	Mg, Ca, Sr Ba	Between locations	Between rivers 100 %	Wells *et al.*, 2003*a*
Newfoundland & Labrador Canada	4 Streams	130 km	*Salmo salar*	2002	Range 22-25 / stream	Mg, Mn, Sr, Ba	Between streams	Between streams 84 – 100 %	Veinott & Porter, 2005
South-east coast Australia	6 Inlets 1-9 sites/inlet	10-760 km	*Pagrus auratus*	2000-2001	Range 2-56 / site	Mn, Sr, Ba	Among bays	85% - 98%	Hamer *et al.*, 2003
Portuguese coast	8 Estuaries	500 km	*Solea solea* *S. senegalensis* *Platichthys flesus* *Diplodus vulgaris* *Dicentrarchus labrax*	2005	Range 30-50/sp.	Li, Na, Mg, K, Mn, Ni, Cu, Zn, Sr, Cd, Ba, Pb	Difference between Species & estuaries	Dependant on Species 70 – 92 %	Vasconcelos *et al.*, 2007
Portuguese coast	8 Estuaries	500 km	*Solea solea* *S. senegalensis* *Platichthys flesus* *Diplodus vulgaris* *Dicentrarchus labrax*	2006	10 / sp. / site	Li, Na, Mg, K, Mn, Ni, Cu, Zn, Sr, Ba, Pb	Difference between 4 species & estuaries	Dependant on Species 6 – 53 %	Vasconcelos *et al.*, 2008
Atlantic coast USA	12 Rivers	1900 km	*Alosa sapidissima*	2000-2002	Range 18-29 / river	Mg, Mn, Sr, Ba	Between rivers	91 %	Walther *et al.*, 2008
Atlantic coast USA	13 Rivers	2700 km	*Alosa sapidissima*	2004	Range 18-59 / river	Mg, Mn, Sr, Ba	Between rivers	93 %	Walther & Thorold 2008
North Wales UK	7	7 km	*Salmo trutta*	2008-2009	Range 11-16 / river	Mg, Mn, Sr, Ba	Between sites	89 %	Ramsay *et al.*, 2011
E-Newfoundland & Canada	4 Rivers 2 estuaries	170 km	*Salmo trutta*	2007-2009	Range 7-16 / river	Mg, Ca, Mn, Zn, Sr, Ba	Between rivers	97 %	Veinott *et al.*, 2012
Newfoundland & Labrador Canada	4 Locations	1100 km	*Gadus morhua*	1998-1999	Range 15-40 Site	Mg, Mn, Sr, Ba	Between groups	66 % 78 % spawning sites merged	D'Avignon & Rose, 2013
Central California	3 Coastal areas Multiple stations	200 km	*Sebastes jordani*	2009	200 total	Mg, Sr, Ba	Between upwelling centre's	-	Woodson *et al.*, 2013
NW- coast UK & E- coast Eire	36 rivers	2,400 km	*Salmo trutta*	2010	Range 15-29 / river	Mg, Mn, Sr, Ba	Between rivers	74 %	Present Study

For example, Wells *et al.* (2003*a*) sampled westslope cutthroat trout (*Oncorhyncus clarki lewisi*) from 3 streams within the Coeur d'Alene River system in Idaho, USA covering a 60 km spatial scale and analysed the microchemistry (Mg, Ca, Sr and Ba) of their otoliths and scales. The accuracy with which individual fish were classified back to their stream of origin was 100% based on otolith chemistry and 82% when using scale chemistry. Studies conducted over greater geographical scales (*i.e.* > 500 km) are less common (see Table 4.22.) but have indicated high classification rates The present study covers one of the largest spatial scales covered to date, encompassing a geographical distance of approximately 2,400 km (1,490 miles) of coastline including the western UK and the east and south-west coast of Ireland and the Isle of Man (see Figure 4.3. for geographical area). To the author's knowledge, the only study covering a larger spatial scale is that of Walther and Thorrold (2008) who studied 13 populations of shad (*Alosa sapidissima*) along the 2700 km Atlantic coastline of the USA.

In addition to varying in the spatial scale over which samples were collected, studies which have used otolith microchemistry to identify fish to source have also varied in the number of source populations that have been sampled. In the present study, 36 separate sources (rivers) were sampled with an estimated average distance between rivers of approximately 72 km (45 miles). To the author's knowledge this is one of the largest data sets used in a microchemistry study to date (as well as covering one of the largest geographical scales). Previous studies using biogeochemical tags have tended to sample from a small number of sources (usually 2 – 10; see Table 4.22.). A meta-analysis of published classification success rates using discriminant function analysis (DFA) shows that there is a tendency for percentage classification accuracy (%) to decrease as the number of sampled sources increases (see Figure 4.18.). This may be because when the number of sources is low, the probability that some fish may be correctly assigned to their source population by chance is greater (see White and Ruttenberg, 2007). The results of the present study do not follow the trend for decreasing classification success with increasing number of source populations (Figure 4.18.) with an average CV-QDFA classification success of 74%. The sample sizes per source (river) in the present study ranged between 15 and 20 with a total sample size of 665 from 36 separate sources. However, the fact that comparable assignment rates were obtained using the Random Forrest technique (Breiman, 2001) and the calculated Cohen's Kappa coefficients (Titus *et al.*, 1984) were high provide confidence that the QDFA analysis is robust and that the

classification of juvenile trout parr back to their source population is not occurring as a result of chance assignments (see Tables 4.8. and 4.9.).

4.7.3. Classification to region based on biogeochemical tags

In comparison to the river assignments (74%), regional classification accuracy was reduced to 66% when the 36 rivers were assigned to their respective sub-regions (see Figure 4.12. and Table 4.13.; 3-6 rivers per sub-region), with classification success ranging from 41% for North Wales to 91% for North Skerries (Table 4.13.). Regional classification relied on the pooling of parr from a number of rivers to determine whether a strong regional chemical tag could be used to assign fish back to origin. The rationale for this was to determine whether the parr baseline could be used to assign marine-caught adults back to region of origin based on the microchemistry of the freshwater region of their otolith. There are several potential criticisms of the sampling strategy employed in the present study; however, this was constrained by factors beyond the control of the present author.

The sampling design for the freshwater baseline work in the Celtic Sea Trout Project involved sampling 50 parr and 50 fry from over 100 rivers in the Irish Sea and Celtic Sea regions, however, since otoliths could only be obtained through terminal sampling, permission was granted to kill a smaller number of fish (*ca.* 25) from 36 rivers distributed within the study region. Therefore, the regional signal is based on pooled data derived from 3-6 rivers per sub-region when clearly there will be more streams / rivers in each sub-region that contain *S. trutta* and produce sea trout. Thus it would be predicted a weaker "regional" signal would be produced from the sampling design. In addition, the distinct chemical signatures observed for some rivers exhibiting *ca.* 100% assignment were probably masked or "diluted" by overlapping other rivers in the sub-region to produce a more mixed (heterogeneous) chemical fingerprint which may have been represented in more than one of the sub-regions resulting in a reduced classification assignment. On reflection perhaps it would have been better to reduce the sample size per river and obtain otolith samples from more rivers per sub-region although in doing so this could have reduced the classification accuracy of fish back to their river of origin.

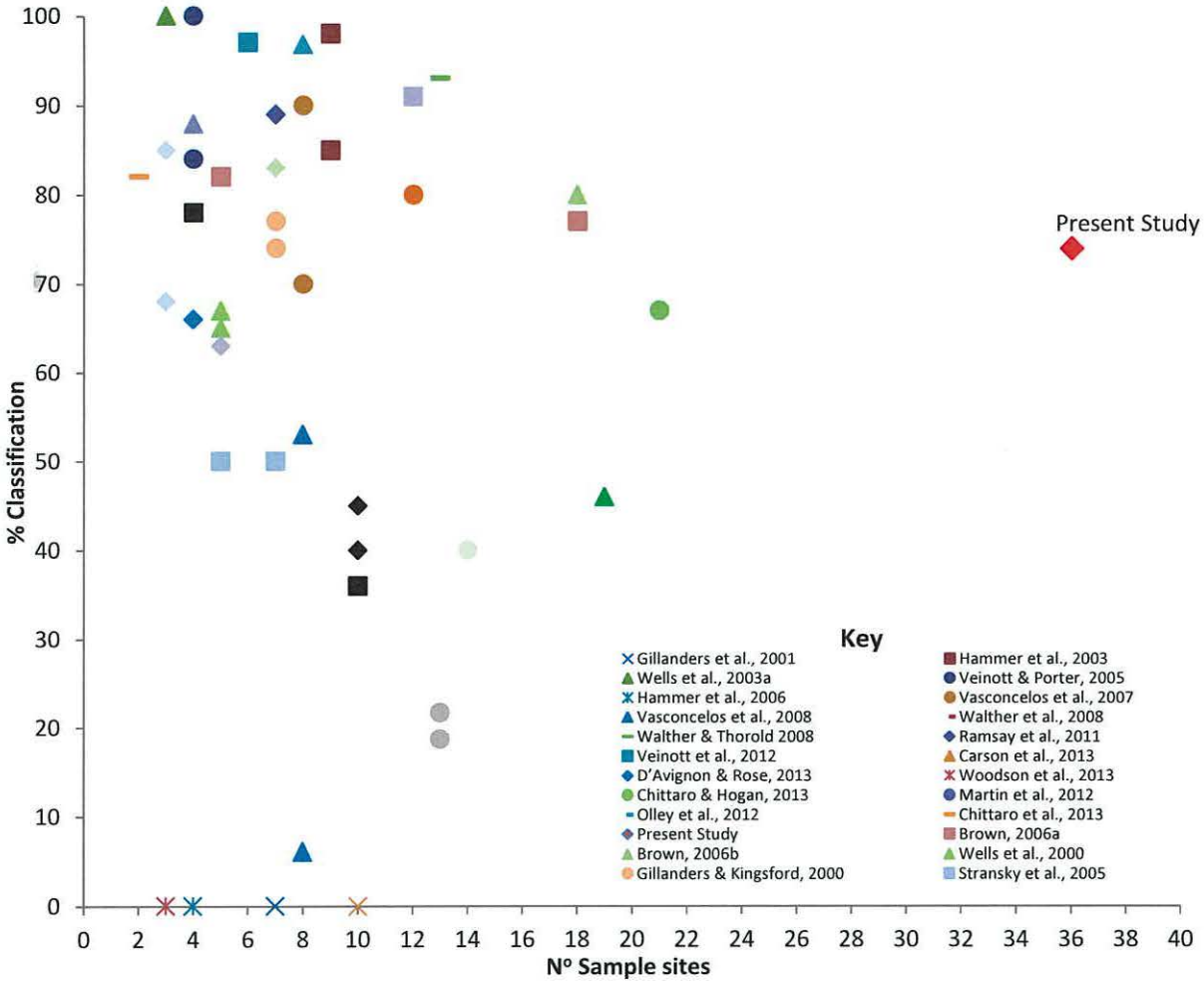


Figure 4.18. Summary of recently published data indicating number of sample locations/sites in regards to percentage classification success using discriminant function analysis and otolith element: Ca ratios.

In the present study, the effect of site pooling (*e.g.* merging rivers to sub-regions) effectively reduced the classification accuracy for the trout parr in this study. Similarly, Gillanders and Kingsford (2003*b*) observed no improvement in assignment accuracy when multiple sites within each estuary were pooled by estuary for 3 species of fish. However, in some studies the effect of site pooling has, conversely, been shown to improve classification accuracy. For example, Fodrie and Herzka (2008) found an increase in classification accuracy for identifying origins of juvenile California halibut (*Paralichthys californicus*) when pooling data from embayments when based on zones (*i.e.* inner vs. outer reaches). Similarly, D'Avignon and Rose (2013) found merging the inshore spawning areas increased their classification accuracy from 66% to 78% when measuring Mg, Mn, Sr and Ba from otoliths removed from Atlantic cod (*Gadus morhua*).

It is possible that pooling samples from a number of sampling sites that are closely co-located in the marine environment may produce a more homogeneous chemical signature compared to more distant sites due to tidal and current mixing of water. However, in freshwater, sampling sites that are separated by < 10 km can present distinctive chemical signatures as a result of the local geology and land use (*e.g.* Ramsay *et al.*, 2011). In addition, reducing the number of potential sources to which a fish needs to be assigned can increase the number of individuals that are allocated correctly, and therefore the classification success, as a result of chance (see White and Ruttenberg, 2007)

4.7.4. Testing the baseline through "blind" assignment:

As discussed in section 4.7.2., using the element:Ca ratios of Mg, Mn, Sr and Ba in trout parr otoliths a biogeochemical baseline was produced where site-specific chemical signatures were observed between the 36 rivers included in the study. In order to test the robustness of this baseline, otoliths from trout parr collected from the same rivers used to establish the baseline were analysed and their putative origin assigned based on the existing freshwater parr baseline. In total 39 fish, randomly selected from the pool of unanalyzed otoliths were assessed and 27/39 fish were successfully allocated to their correct river of origin. These results provide greater confidence in the baseline assignments recorded in the present study. To the author's knowledge there have been no other studies using the same approach to assign "blind" run samples to source using a

biogeochemical baseline, especially with such a large sampling data set covering a large geographical area.

4.7.3. Adult classification: Marine growth

Classification success to the five marine zones of capture (MZs 6, 10, 13, 14 and 23; see Figure 4.19.) using the marine growth phase of the otolith for adult sea trout was poor with only 33% of adult trout correctly assigned to their capture zone using otolith concentrations of 7 elements: Na, Mg, K, Mn, Zn, Sn and Ba (See Figure 4.19.). However, although classification assignment was low, individual classification of adults caught from marine zones MZ-10 and MZ-06 indicated a classification accuracy of 41% and 42% respectively back to their zones of capture (see Table 4.19.), with the remaining marine zones MZs 13, 14, and 23 (see Figure 4.19.) indicating a more mixed distribution of adult trout within each zone. The lack of distinct marine phase chemical tags between adult sea trout caught in each marine zone indicates that either (1) the water chemistry in the Irish Sea is fairly homogenous and the water within each marine zone does not have a distinctive chemical signature or (2) spatial heterogeneity in water chemistry may exist in the Irish Sea between some / all marine zones but individual sea trout undertake extensive feeding migrations around the Irish sea and thus the integrated signature obtained from the entire transect of the marine phase of the adult otolith does not share a strong similarity to the marine zone of capture.

The period of marine residency and the marine movement patterns of sea trout are little understood, with sea trout spending anything from a few months up to 3 years at sea before returning to spawn (Klemetsen *et al.*, 2003), with distances travelled by individual fish likened to an energy trade off where they will only go as far as required to gain the maximum potential benefits from sea migration (Solomon, 2006). The actual movement patterns of sea trout within coastal waters and further afield in open water (*i.e.* marine migration) are little understood (Gargan *et al.*, 2006). It has been assumed that movement patterns tend to be more localized, with most adult trout migrating no further than the coastal waters near to their natal river where they may return to overwinter (Gargan *et al.*, 2006) and a recent study by Veinott *et al.* (2012) would confirm this assumption.

However, there can be considerable variation between individuals in terms of their

life history strategy (*i.e.* how long they stay at sea and where they go; Klemetsen *et al.*, 2003) and previous studies have shown that sea trout have the capacity to migrate longer distances (see Berg and Berg, 1987; Okumuş *et al.*, 2006 and references therein) although most, would appear to refrain from doing so (Gargan *et al.*, 2006; Veinott *et al.*, 2012).

By ablating the last period of growth on the adult trout otolith it can be assumed that the most recently deposited aragonite will be measured and it will produce an estimate for any chemical signal derived from the most recently visited marine environment (see Veinott and Porter, 2005). If this assumption is correct for the adult trout otoliths from the present study then measurements of the marine growth signal have indicated a somewhat mixed biogeochemical signal. This could suggest extensive pan-Irish Sea migrations, however, the 'spatial homogeneity' hypothesis cannot be discounted as the water chemistry of the Irish Sea may be a relatively homogeneous environment with respect to its elemental composition (see Vincent *et al.*, 2004) and as such adult sea trout may reside in a particular area of coastal water which may or may not be near to their natal rivers but no distinctive chemical tag may be deposited within the otolith (Thresher, 1999; Gillanders *et al.*, 2001; Vasconcelos *et al.*, 2007).

Such an occurrence of this type of phenomenon has been suggested by Gillanders *et al.* (2001) who found a lack of elemental differences within the otolith chemistry of two-banded bream (*Diplodus vulgaris*) between locations sampled along the south-west coast of Spain. The results of Gillanders *et al.* (2001) reported spatial homogeneity in otolith chemistry for bream sampled at different locations and the lack of chemical differentiation between locations was suggested to be due to very few differences in water chemistry at these sites as there were no major rivers found within the 400 km of their study area and rainfall in the region was very low (*ca.* 300 mm $year^{-1}$). Therefore, any environmental influences due to freshwater runoff and its effects on the ambient water chemistry (*e.g.* temperature, salinity and trace element concentrations) within the study area were considered to be minimal (Gillanders *et al.*, 2001) resulting in a more uniform biogeochemical signal.

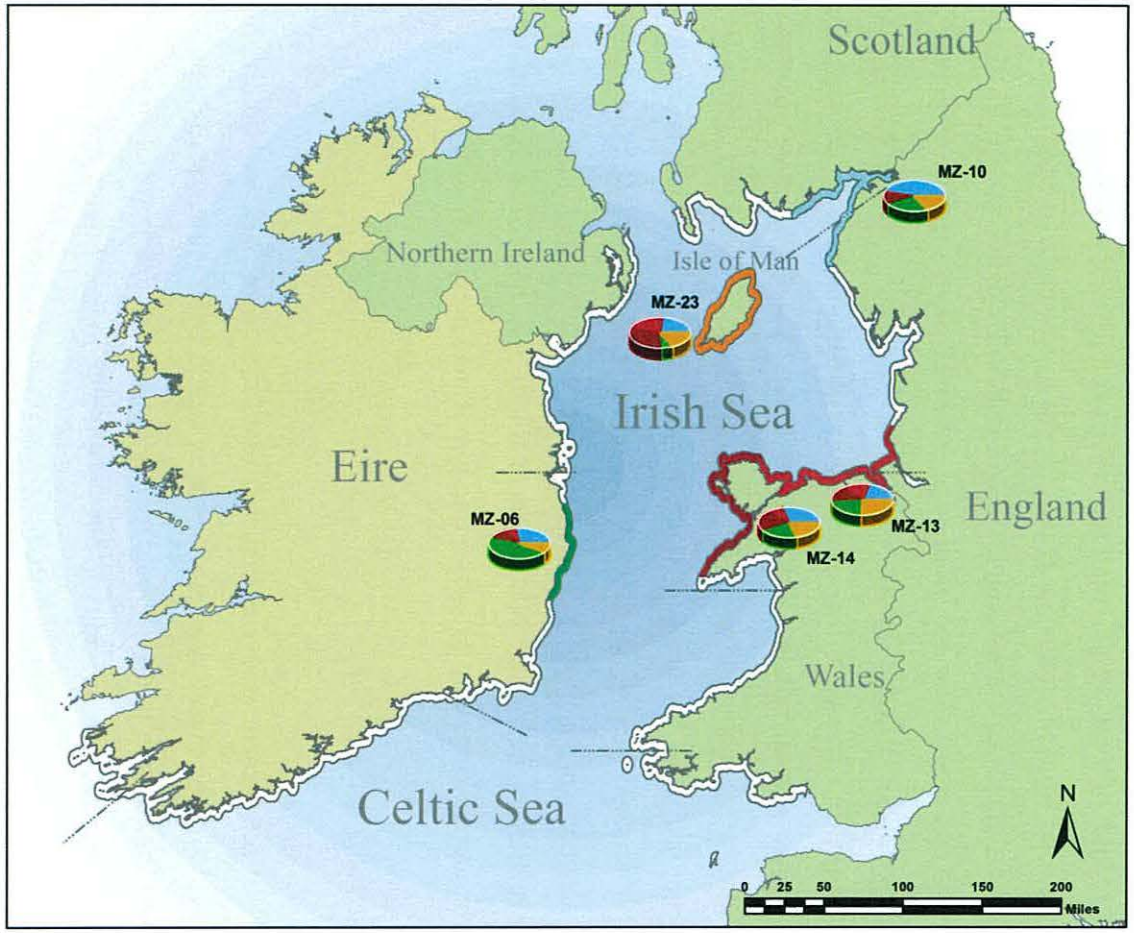


Figure 4.19. Map of the Irish Sea indicating the 5 marine zones sampled for adult sea trout *S. trutta*. Adult sea trout were assigned to their marine zone of capture using CV-QDFA and the Log_{10} elements Na, Mg, K, Mn, Zn, Sn and Ba. Pie chart segments represent each of the 5 marine zones.

4.7.4. Adult classification: Freshwater growth

Based on previous work suggesting that adult sea trout remain in coastal waters close to their natal river and do not undertake extensive migrations (Gargan *et al.*, 2006; Veinott *et al.*, 2012), it would be expected that adult sea trout caught in a particular marine zone would classify to the sub-region adjacent to that marine zone based on the chemistry of the freshwater section of their otolith (see Figure 4.4.) and using the freshwater parr baseline. For example, one would hypothesise that adult sea trout caught in MZ10 should classify back to SW Scotland and those caught in MZ6 should classify to one of the two sub-regions on the east coast of Ireland (north or south of the Skerries). However, based on this hypothesis overall classification accuracy was poor with only 17.5% correctly classifying to the sub-region(s) adjacent to their marine zone of capture (see Figure 4.20. and Table 4.23.). There are several possible explanations for the poor assignment success of adult fish to the freshwater region adjacent to their marine zone of capture: (1) the freshwater baseline established in the present study may not be an accurate descriptor of the freshwater microchemistry signal for each sub-region, (2) issues of temporal stability may affect the accuracy of the freshwater microchemistry baseline or (3) the baseline may be accurately predicting freshwater sub-region of origin and the results show that adult sea trout may be undertaking more extensive migrations than previously thought.

a) *The baseline is not accurate*

The results of the present study have shown that 74/66% of the parr could be correctly classified back to their river / region of origin: given that the present study comprises one of the largest data sets in terms of geographical coverage and number of sources, this is a high classification success. In addition, 69% of the fish in the "blind" allocation were also correctly assigned to their river of origin. However, there are clearly more than 36 sea trout-producing streams / rivers in the Irish Sea region, a geographical area covering approximately 2,400 km of coastline (UK: Solomon, 1995; Harris; 2006; Ireland: McGinnity *et al.*, 2003; Isle of Man: Anon, 2012). The exact number is not known and previous studies have tended to only identify the major catchments that are known to contain migratory salmonid populations (*i.e.* including Atlantic salmon or sea trout).

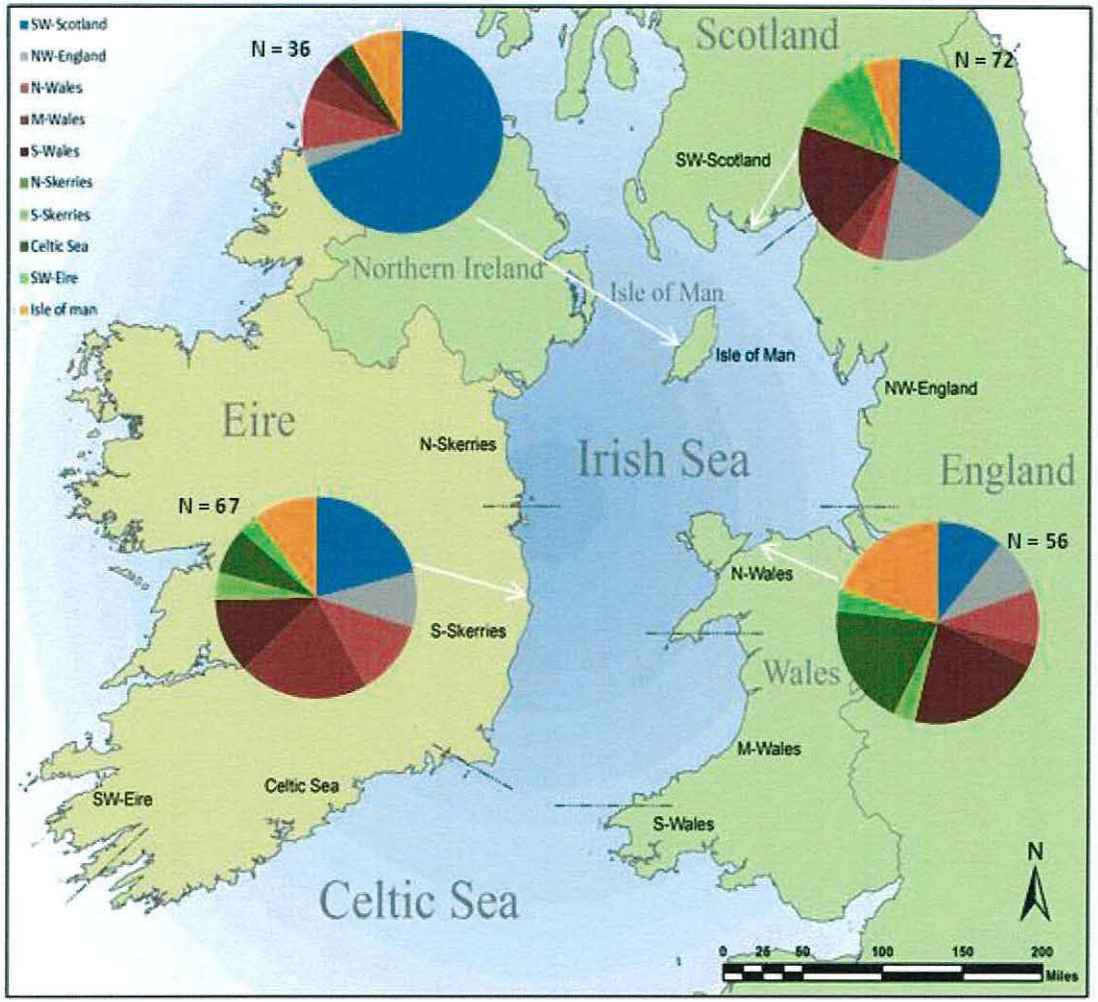


Figure 4.20. Classification accuracy of marine caught adult sea trout to their sub-region of origin using the freshwater parr base-line created from Mg, Mn, Sr and Ba. Pie charts indicate sub-region to which adult trout were assigned using their freshwater residency period (see Figure 4.4.).

For example, 261 discrete migratory salmonid producing rivers have been listed in Ireland (McGinnity *et al.*, 2003), over 80 rivers in the western UK (*i.e.* including south-west Scotland, north-west England and Wales; Solomon, 1995; Harris, 2006) and 8 major and 18 smaller rivers and tributaries found in the Isle of Man (Anon, 2012). In addition, it is important to note that small coastal streams can also be important and productive sea trout habitat (Jonsson *et al.*, 2001; Klemetsen *et al.*, 2003) and so the true number of potential sources of sea trout in the Irish Sea region is likely to number in the hundreds. This phenomenon of unidentified systems not included in those sources contributing to the sampled data has been termed as "ghost" populations (see Veinott *et al.*, 2012). It is important to note that a major restriction on the present study was the limitation placed on the number of fish that could be retained for otolith microchemical work. The licences to retain juvenile trout parr issued by the relevant agencies in the UK and Ireland only allowed 20-25 parr to be killed from limited number of rivers within the sampling area (*i.e.* the Irish and Celtic Seas). Therefore, because of the restrictions imposed, samples were only collected from the main sea trout producing rivers within each of the 10 sub-regions based on the assumption that these rivers would be more likely to be represented in any marine catches of adults. It should be noted that this could result in weak regional signals being observed. Although one must be aware of the potential limitations of the composition of the baseline, it must be stressed that a high classification accuracy was achieved (despite the large number of source populations and large spatial scale of the study) and that it provides a suitable baseline against which to try to allocate marine-caught adult sea trout.

b) *The baseline is not stable*

A second reason why adult sea trout caught in a particular marine zone may classify poorly to their adjacent freshwater sub-region could due to temporal instability in the biogeochemistry of the freshwater baseline. Although they were not aged within the time frame of the present study, the parr collected to establish the freshwater baseline in 2010 were likely to be 1 and 2 year old fish based on their size frequency distribution (see Table 4.2.). This would mean that these fish were born in 2008 or 2009 and so the time period 2008-2010 would be included in the chemistry used to establish the freshwater baseline. Adult sea trout were caught in 2010-2012 (with the majority caught in 2010-11;

see Table 4.3.). It was not possible to age these fish within the timeframe of the present study in order to determine for each fish the period of time spent in freshwater and in the marine environment and so determine the calendar years when each fish was resident in freshwater. Therefore, it is possible that some fish may have been resident in freshwater pre-2008, although it is thought that this is unlikely as given the size of the sea trout most fish are likely to have spent 1-3 years at sea prior to capture. However, the results of Chapter 3 and other published studies (Rooker *et al.*, 2001; Gillanders, 2002; Kerr *et al.*, 2007; Walther *et al.*, 2008) have shown that although long term temporal stability in chemical tag may not occur, temporal stability between consecutive years is often present. Therefore, it is likely that even if the freshwater residency period for some marine-caught adults does not include the calendar years 2008-2010 (*i.e.* they migrated to sea prior to 2008) then they would have been resident in freshwater in the years immediately preceding (*i.e.* 2006-2007) and temporal stability is more likely to be present. Therefore, it is concluded that temporal instability of the freshwater baseline is unlikely to explain the poor classification of adult sea trout caught in a given marine zone to the adjacent freshwater sub-region.

c) Fish may be migrating further than previously thought

Based on previous work which suggested that adult sea trout tend to remain in coastal waters close to their natal rivers to feed (Pemberton, 1976; Elliott, 1994; Knutsen *et al.*, 2001; Klemetsen *et al.*, 2003; Jonsson and Jonsson, 2011*a*; Jensen and Rikardsen, 2012), it was hypothesized in the present study that fish caught in a particular marine zone would classify back to the freshwater sub-region adjacent to that marine zone, for example, fish caught in MZ10 would tend to classify back to southwest Scotland and fish caught in MZs 13 & 14 would tend to classify back to north Wales. If this assumption is correct then the classification from marine zone to adjacent freshwater sub-region was poor at 17% (Table 4.21.). However, if the classification to freshwater sub-region of origin is correct then the results suggest that some adult sea trout may be undertaking more extensive migrations than previously thought.

Table 4.23. reinterprets the classification data presented in Table 4.21. to determine what percentages of fish putatively derived from each freshwater sub-region are captured in each Marine Zone.

Table 4.23. QDFA Predicted Classification of adult marine caught sea trout *Salmo trutta* using the freshwater growth phase to their region of origin from the biogeochemistry baseline established using Mg, Mn, Sr and Ba. Adult fish were assigned based on the probability of belonging to a particular region. Correct numbers of parr per region are shown with percentage classification of sub-region to marine zone (row total %) highlighted in bold. MZ-13 and MZ-14 are combined. Sub-regions highlighted represent the colours used to indicate the movement patterns of fish in Figure 4.21.

	Marine Zone				
Sub-Region	**MZ-10**	**MZ-13-14**	**MZ-06**	**MZ-23**	**Row Total**
SW-Scotland	25 (**36%**)	6 (**9%**)	14 (**20%**)	25 (**36%**)	70
NW-England	13 (**52%**)	5 (**20%**)	6 (**24%**)	1 (**4%**)	25
N-Wales	3 (**16%**)	5 (**26%**)	8 (**42%**)	3 (**16%**)	19
M-Wales	3 (**14%**)	2 (**10%**)	14 (**67%**)	2 (**10%**)	21
S-Wales	14 (**40%**)	12 (**34%**)	8 (**23%**)	1 (**3%**)	35
N-Skerries					0
S-Skerries	5 (**50%**)	2 (**20%**)	3 (**30%**)		10
Celtic Sea		11 (**65%**)	5 (**29%**)	1 (**6%**)	17
SW-Eire	5 (**56%**)	2 (**22%**)	2 (**22%**)		9
Isle of man	4 (**16%**)	11 (**44%**)	7 (**28%**)	3 (**12%**)	25

Using these data it is possible to derive the putative movement patterns within the Irish Sea for fish from each freshwater sub-region. Figure 4.21. presents these putative movement patterns for sub-regions where the sample size of fish is *ca.* 20 or larger and presents the movement patterns where ≥ 20% of the fish are caught in a given marine zone. These data would indicate that the movement patterns of adult sea trout in the Irish Sea could be more extensive than previously suggested and that migratory patterns may in some way be related to sea surface water currents (Figure 4.22.). The movement patterns of adult sea trout putatively derived from SW-Scotland (Figure 4.21.A) indicated shorter migratory patterns with most adults remaining within the coastal waters of the Solway (36%) or migrating to the coastal waters off the Isle of Man (36%), a distance of approximately 76 km (calculated as a linear distance, see Table 4.24.).

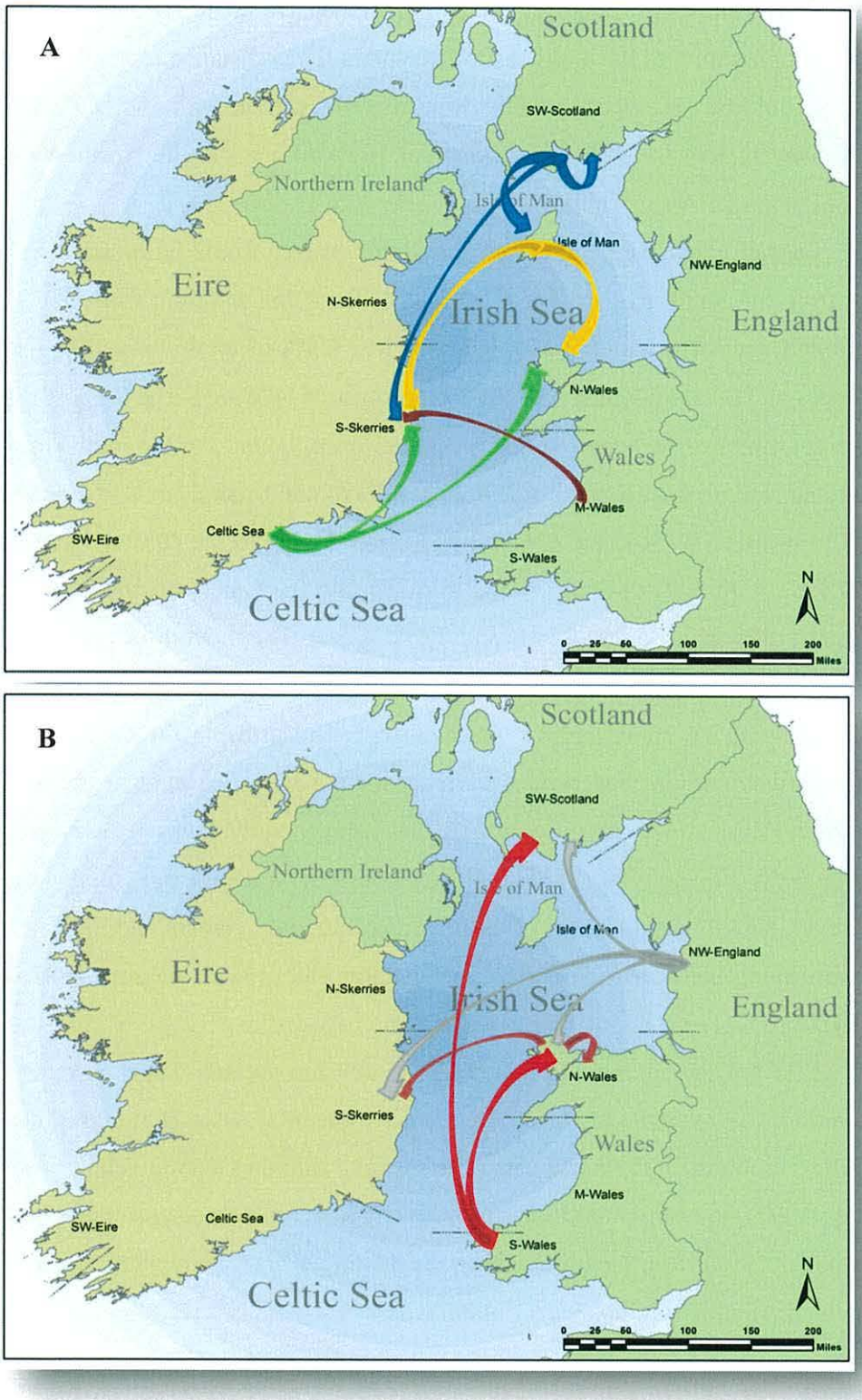


Figure 4.21. Estimated putative movement patterns of adult sea trout *Salmo trutta* within the Irish Sea for fish from each of the freshwater sub-regions using the QDFA classification data presented in Table 4.23. Data is taken where sample size is *ca.* 20 or larger and where ≥ 20% of adult fish were caught in a given marine zone. Arrows are colour coded to identify sub-region (see Table 4.23).

However, some distances were more extensive with 20% of adults from SW-Scotland migrating to the east coast of Ireland, a linear distance of some 238 km. Similar results could be observed for adults from the Isle of Man with 28% indicating a similar movement pattern to Ireland (a distance of 162 km) and 44% to North Wales (122 km) (see Figure 4.21.A and Table 4.23. and Table 4.24.).

Further extensive patterns of migration however, could be inferred for those adult fish from the Southern Irish Sea (Figure 4.21.B). Adult sea trout from South Wales also exhibited extensive patterns of migration, with 23% of adults travelling 117 km to S-Skerries and 34% travelling 157 km to the coast of N-Wales. The longest distance adult sea trout migrated was approximately 355 km from S-Wales to the south western coast of Scotland (Figure 4.21.B and Table 4.24.). 52% of adult fish from NW-England tended to move towards SW-Scotland (71 km) and 20% moving to the coastal waters of N-Wales (127 km), with 24% of adults found to move into the waters off the south east coast of Ireland (167 km), (Figure 4.21.B and Table 4.24.). The movement patterns of adult sea trout within the Irish Sea and highlighted in Figures 4.21. may be the result of the adult fish following the main surface water currents and utilising these currents to assist in shoreward migration. Sea surface currents for the Irish Sea tend to travel up from the south and circulate clock-wise around the inshore coastal waters of North Wales and South-West England (see Figure 4.22.). Surface waters from south west Scotland however, tend to show an anti-clock-wise directional movement and indicate waters tend to flow along the coast of Ireland (Lee and Ramster, 1981; Dickson, 1987; Humphries, 2004).

It is not uncommon for salmonids to use the surface water currents to assist in migration. For example, surface currents are thought to assist the migrations patterns of Pacific salmonids in the north east (*e.g.* sockeye salmon Oncorhynchus nerka; Thomson *et al.*, 1992) and north west Pacific (*e.g.* chum salmon Oncorhynchus keta; Groot and Margolis, 1991). Similar observations by Holm *et al.* (2000) were observed for post-smolts of Atlantic salmon (*Salmo salar*) where post-smolts were found to follow the main surface currents northward towards the Norwegian Sea. Similarly, a study by Lacroix (2013) with the aid of pop-up satellite archival tags identified changes in oceanic migration of Atlantic salmon kelts transported using the North Atlantic current from three distinct Canadian populations.

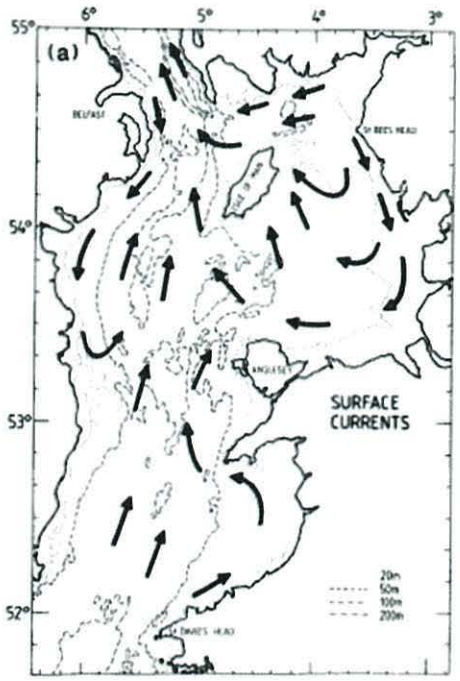


Figure 4.22. Map indicating the near sea surface current directional flow within the waters of the Irish Sea. (Taken from Dickson.1987).

Table 4.24. Calculated linear distances (in km) of adult sea trout *Salmo trutta* putative movement patterns within the Irish Sea for fish from each freshwater sub-region derived from Table 4.7.2.

Region	Assigned Region	Linear distance (km)
SW-Scotland	Isle of man	76
	S-Skerries	238
NW-England	SW-Scotland	71
	N-Wales	127
	S-Skerries	167
N-Wales	S-Skerries	39
M-Wales	S-Skerries	50
S-Wales	SW-Scotland	355
	N-Wales	157
	S-Skerries	117
Celtic Sea	N-Wales	159
	S-Skerries	120
Isle of man	N-Wales	122
	S-Skerries	162

A possible correlation between sea surface currents and the migratory movement patterns of adult sea trout could be observed in the present study (see Figures 4.21. and 4.22.). If we are to assume that adult sea trout are showing more extensive movement patterns than previously thought within the Irish Sea then more information is required to corroborate the findings presented here. As part of the larger C.S.T.P. project, a complimentary genetics project using microsatellite markers has also suggested that fish derived from particular sub-regions of the Irish Sea may be undertaking extensive pan-Irish Sea migrations. The coupling of genetic analyses and otolith trace elements could provide better insights into understanding movement patterns of adult trout (see Perrier *et al.*, 2011) and may strengthen the results presented here and support the hypothesis that adult sea trout undertake greater more extensive marine migrations than previously suggested.

4.7.5. Conclusion

The use of biogeochemistry to infer freshwater natal origin of *Salmo trutta* parr and to identify individual parr to their natal rivers using otolith microchemistry has been shown to be a reliable and consistent tool in this present study. Moreover, this study has shown that robust freshwater baselines can be established with high classification success of juvenile trout parr samples (which were run blind) to their natal river using the four elements Mg, Mn, Sr and Ba. Additionally, if the chemical signatures identified can be shown to be temporally stable with little variation over two or more years, then the identification of the recruitment origins of adult migratory trout (*i.e.* sea trout) populations may be inferred based on the natal period of growth in their otoliths. Using the freshwater baseline to assign adult sea trout to their putative freshwater region of origin using their freshwater period of growth has indicated a possible conflict in the understanding of adult migratory patterns during their period at sea. In contrast to the accepted view that adult sea trout remain in coastal waters close to their natal rivers, the results of the present study suggest that the migratory patterns for sea trout in the Irish Sea may be more extensive. In conclusion, it is clear that our understanding of the migratory history of adult sea trout is limited and that further work is necessary to elucidate the movement patterns of this species in the marine environment.

Chapter 5

General Discussion

5.1. General Discussion

Since its first applications discussed in six papers prior to 1980, the use of trace element microchemistry in the scales and otoliths of fishes has developed into a major tool for studying the ecology, life histories and movement patterns of fishes (Campana, 1999; Elsdon *et al.*, 2008; Sturrock *et al.*, 2012). The application of the technique continues to grow with an exponential increase currently observed in the numbers of papers published each year (Sturrock *et al.*, 2012, Chapter 3 section *3.1*). However, although its application is becoming widespread, its validity is based on a number of assumptions that have not been rigorously tested, namely that the chemistry of the scale / otolith remains "locked in" and cannot be altered by the various processing stages prior to measurement (*e.g.* methods of killing, transportation or storage and methods of preparation in the laboratory) and that any site-specific "unique" chemical tag remains stable over time and distinguishable from other sources. The aim of this thesis was to address these assumptions (Chapters 2 and 3) and to further develop the application of the technique in studying the movement patterns of fishes (Chapter 4).

This thesis addressed four main aims. Firstly, to determine the effects of methods of dispatch and transportation and storage duration [short (*i.e.* weeks) and long term (*i.e.* months to 1 year)] on the trace elemental concentrations incorporated within sea bass *Dicentrarchus labrax* otoliths. Secondly, to assess the temporal stability of the trace element chemical tag within the otoliths of juvenile plaice *Pleuronectes platessa* collected from two recognised nursery grounds in North Wales over an extended time period (2004-2010). Thirdly, to assess the use of trace elements as natural biogeochemical markers to assign brown trout *Salmo trutta* parr back to their natal rivers using otolith microchemistry and using a large number of source populations over one of the largest spatial scales studies to date. Fourthly, to assess the efficacy of this biogeochemical baseline to assign marine-caught adult sea trout back to their geographical region of freshwater origin.

5.2. Effects of anaesthesia, transportation and storage duration on otolith trace element composition

The effects of possible contamination caused by *post mortem* handling and during sample preparation (*e.g.* otolith extraction and cleaning) are a constant concern for researchers

wishing to measure trace and ultra-trace elemental concentrations in fish otoliths / scales using techniques such as inductively-coupled plasma mass spectrometry (ICP-MS). Problems arising through contamination have been highlighted by a number of studies (*e.g.* Thresher, 1999; Rooker *et al.*, 2001; Swan *et al.*, 2006) who have put forward protocols to be implemented for decontamination to reduce these sources of error (see also Campana, 1999 for a review of cleaning protocols). However, no studies to this authors knowledge have measured the possible effects on trace element composition within otoliths for the differing methods used to dispatch, transport and ultimately store the whole fish frozen for both short (*i.e.* days-weeks) and long-term (*i.e.* months – 1 year) storage durations.

The results of Chapter 2 show that neither the anaesthetic used to kill the fish nor the transportation method implemented from sampling location to the laboratory nor the storage duration had any significant effect on otolith elemental concentrations of Na, Sr and Ba. Similarly, although both Mg and K were reported to be significantly different after a storage duration of 6 months when compared to the remaining storage periods, this was thought to be the result of the data distribution and reflected a Type 1 error. Otolith concentrations of Mn were affected by storage duration with a significant difference observed between the shortest and longest storage period (*i.e.* 1 day and 12 months). The experimental design Chapter 2 was established to mimic the storage time periods most likely to be observed within a normal (*i.e.* 3 year) research programme. Although it would have been beneficial to measure storage durations in excess of one year, this was not feasible within the time frame of this thesis. However, this is an important point given the fact that samples were analysed in Chapter 3 that had been stored for up to 7 years prior to measurement. The results of the present study have shown two of the major elements used in biogeochemical tagging, Sr and Ba (see Muhlfeld *et al.*, 2005; Veinott and Porter, 2005; Ramsay *et al.*, 2011) were not affected by, and were found to be resilient to the most commonly used methods for fish dispatch, transportation and freezer storage duration. Both Sr and Ba have been previously identified as two elements which will substitute for Ca within the crystal lattice of the otolith matrix (Speer, 1983; Kalish, 1989; Rooker *et al.*, 2001) and are therefore not as susceptible to leaching or contamination affects which has been observed for some elements (see Milton and Chenery, 1998; Proctor and Thresher, 1998; Rooker *et al.*, 2001; Hedges *et al.*, 2004).

In conclusion, the results of Chapter 2 in this thesis have shown that the methods used in the thesis to dispatch fish, transportation method and storage durations in the

freezer of up to one year do not affect the chemistry of the otolith with respect to the elements Sr, Ba, Mg and K. Fish samples retained and stored in this way can be utilised in studies to discriminate between populations / sources of fish (*e.g.* Gillanders, 2005; Wells *et al.*, 2000; Walther and Thorrold, 2008; Walther *et al.*, 2008; Ramsay *et al.*, 2011, 2012) using otolith microchemistry and solution-based ICP-MS. However, there does appear to be an effect of long-term storage duration (1 year) on otolith concentrations of manganese. It is recommended that future work examine whether this result is consistent (*i.e.* not a Type 1 error as observed with Mg and K) and whether longer storage durations further influence otolith Mn concentrations. In the interim, it is recommended that storage periods of < 12 months are used in otolith microchemistry studies involving the use of Mn.

5.3. Effects of temporal variability on otolith trace elements

Although it is possible to measure trace and ultra-trace elemental concentrations incorporated within fish otoliths using techniques such as ICP-MS, understanding and interpreting the data in what can be considered as a very dynamic and active environment (*e.g.* freshwater, estuarine or marine waters) requires a choice of sampling regimes designed specifically to investigate both spatial and temporal changes. As a result of seasonal cycles, otoliths in fish can be subject to inter-annual variations in their elemental composition which could confound the interpretations observed on a spatial scale (Gillanders, 2002). It is becoming apparent that problems in data interpretation can arise as a result of the dynamic and variable nature of the aquatic environment where water chemistry can be influenced by factors such as changes in temperature and salinity which can affect the solubility and concentrations of elements and the rates at which these elements are incorporated into the otolith matrix (Martin and Thorrold, 2005; Lin *et al.*, 2007). In order to fully understand the effects of these variables would require a sampling design to take into account not only potential daily changes (*i.e.* tide cycles see Elsdon and Gillanders, 2006*b*) in water chemistry but also weekly and monthly changes as a result of seasonal changes in temperature and the seasonal influx of water (*e.g.* rainfall, run-off etc.). Furthermore, it would be advantageous to take into account longer periods of time in sampling regimes that would cover several years if we are to fully address questions of temporal stability of unique site-specific chemical tags and be confident in our results looking into spatial variability and the application of this technique to identify

origins and movement patterns. (Gillanders, 2002; Elsdon and Gillanders, 2006*b*; Elsdon *et al.*, 2008). Clearly, such an approach is beyond the capability of most studies, in terms of the resources required (finance and time), however, a few "benchmark" validation studies to fully understand the factors influencing temporal changes in otolith chemistry at any given site and the temporal stability the chemistry of this site, with reference to other potential source locations, would be highly recommended. The results presented in Chapter 3, were an attempt to address this issue, however, during the statistical analysis of the data it became apparent that the aim of addressing temporal stability over a long time period (2004-2010) were confounded by issues of sampling design.

The results presented in Chapter 3 indicate that temporal stability was evident for certain elements over time scales of several years, for example for Mg (Llanfairfechan) and Na and Sr (Llanddona) over a 3 year period (2004-2006), increasing to 4 years (2004-2006) for Na and Ba (Llanfairfechan) and K and Ba (Llanddona). As described in Chapter 3, the strongest evidence for temporal stability were observed for the earlier years (2004-2006) in the time series which corresponded to those years where the most monthly periods of sampling took place and it is highly probable that this has produced the most reliable average annual elemental signature for each site. Conversely, the years where sampling frequency was restricted to only 1 month (2009-2010 both sites) or where sample sizes were small (2007 both sites), elemental concentrations for Na, Mg and Ba at Llanfairfechan and Na, K, Sr and Ba at Llanddona were found to be more variable. The samples analysed in Chapter 3 were 1+ plaice that had been collected as part of a previous PhD project focusing on the growth rates of 0+ plaice on the two nursery grounds (Al-Rashada, 2009 unpublished data). Sampling during every month was not possible as a result of weather and sea state conditions and 1+ fish were only opportunistically retained as bycatch rather than specifically targeted. Therefore, an unbalanced experimental design resulted with fewer sampling events occurring in some years (especially later years when the survey was continued as a "side activity" after completion of the PhD, accounting for the absence of sampling in 2008), the same months not being sampled in each year and variable samples sizes between months / years. All of these factors presented problems in the statistical analysis and made it difficult to unequivocally address the issue of temporal stability.

To address this problem, if time and cost were not limiting factors, in any future study samples should be collected using a sampling design which incorporates a range of temporal scales (*i.e.* both inter and intra annual) that would reflect the chemistry

incorporated within the otoliths. The use of nested designs or "Hierarchal" designs (see Underwood, 1997) combined with statistical analyses of a nested design to enable the interpretation of those results may provide a more robust approach in understanding and addressing the question of temporal stability (Elsdon and Gillanders, 2006*b*). This would involve collecting samples over a range of time scales including on a daily (*i.e.* over two tidal cycles) or weekly basis (incorporating daily tidal cycles) for up to one year (to assess short term intra-variability) and again on a monthly scale for a designated yearly sampling regime (*e.g.* 3-5 years) for long-term inter-annual analysis.

However, it may not be always feasible, and may be logistically difficult to implement such a sampling design, see Olley *et al.* (2011). If we remove the major requirement of where to store all the samples during this sampling regime (*e.g.* 3-5 years) and the cost incurred to run the samples on the ICP-MS, we then have to take into account sample numbers, time required (weather permitting) to collect and process the samples and the legal / ethical procedures involved. The sample sizes utilized by various authors in fish microchemistry studies looking at temporal variation in otolith chemistry vary considerably, *e.g.* from between 10-20 fish per collection site (Gillanders, 2002; Elsdon and Gillanders, 2006*b*) to in excess of a 100 (minimum of 100 samples see Campana *et al.*, 2000). If we were to sample on the conservative side and retain 10 fish per sampling this would equate to 120 fish per year just for a single monthly sample collection and over a protracted maximum 5 year period would therefore increase the numbers to 600 fish samples. If we wish to utilize two sites (as was conducted for the present study) this would increase to 1,200 samples in total to assess inter-annual variability in and between two separate sites. Similarly, to conduct the analyses required to assess intra-annual temporal stability, *e.g.* daily and weekly sampling (to incorporate tidal cycles) over a year, the required number of fish samples would run into the thousands which would be logistically and financially difficult to implement. In addition, if working on wild fish populations there would be a requirement to obtain special dispensation from local fisheries authorities to catch undersize juvenile fish with the criteria required by Elsdon and Gillanders (2005) (residency time > 20 days). These numbers may be of a major concern to those fisheries organisations issuing the license.

To summarise, if we are to assess the chemical variability of a site and understand any temporal variation then we must have a clear understanding of the dynamic environment and the differences in the chemistry of the water varying time scales. Although the present study indicated some temporal stability for both nursery sites

between 2-3 years, our understanding of temporal variability and the variation in water chemistry with regards to fish movement is still limited and future work must encompass these variables if these movements are to be related to habitat residency over time (Elsdon *et al.*, 2008).

5.4. The use of otoliths to assess spatial variability and determine natal zones

The understanding of movement patterns and natal origins of fish populations to identify discrete nursery grounds and ascertain natal origins of adult fish is fundamental if we are to effectively manage commercial and non-commercial fish species. The microchemistry of calcified structures in fishes (*i.e.* otoliths and scales; Ramsay *et al.*, 2011) is developing into a valuable tool in identifying origins and movement patterns (Elsdon and Gillanders, 2003*a*; Elsdon *et al.*, 2008; Tanner *et al.*, 2012; Veinott *et al.*, 2012). A multi-elemental signature approach is usually adopted and the most common trace elements measured in fish otoliths and most commonly applied to biogeochemical tagging studies are Mg, Mn, Sr and Ba (see Muhlfeld *et al.*, 2005; Veinott and Porter, 2005; Ramsay *et al.*, 2011). To date, most published studies have focused on a small number of source populations and / or over a small spatial scale (see section 4.7.2 in Chapter 4). In Chapter 4 the spatial variability in biogeochemical tag was examined for 36 rivers sampled from the western UK and Eastern Ireland (see Chapter 4 Figure 4.3.) to elucidate stock discrimination between rivers for juvenile *Salmo trutta* parr. The study presented in Chapter 4 is one of the largest studies conducted to date in terms of the number of source populations (n=36) and the spatial scale of its coverage (*i.e.* covering an entire coastal shelf sea region with a coastline of *ca.* 2500 km). In addition, two novel extensions of the otolith microchemistry baseline technique were included in Chapter 4. Firstly, the robustness of an established freshwater baseline was tested using trout parr obtained from the same source populations but not involved in establishing the baseline. Secondly, the established freshwater baseline was used to try to assign marine-caught adult sea trout back to their putative natal region of origin using the chemistry of the otolith corresponding to their freshwater residence period. To the author's knowledge the first extension of the microchemistry technique presented in Chapter 4 has not been attempted before and the second extension to the technique has only recently been applied within the lifetime of this Ph.D. project (Olley *et al.*, 2011; Veinott *et al.*, 2012; Veinott and Porter, 2013).

The use of Mg, Mn, Sr and Ba measured from juvenile trout parr otoliths sampled from 36 rivers in the western UK and Eastern Ireland enabled the classification of individual trout parr back to their natal rivers with a classification accuracy of 74%. Using trout parr obtained from the same source populations but not involved in establishing the baseline for the present study resulted in 27/39 "blind" run trout parr correctly assigned to their natal rivers. The high classification accuracy of the "blind" run samples using this biogeochemical baseline indicate the robustness of this technique further giving credence to the use of the established baseline in attempting to assigning marine-caught adult sea trout back to their putative natal regions.

Classification of marine-caught sea trout however, was poor, with less than 20% of those adults classifying back to their region of origin. As mentioned in Chapter 4, a major restriction on the present study was the limitation placed on the number of fish that could be retained for otolith microchemical work. Licences to retain only 20-25 parr issued by the relevant agencies (UK and Ireland) and the limited number of rivers within each of the 10 sub-regions may be one of the contributing factors in a weak regional signal being observed for the present study. Furthermore, the limitations imposed on sample retention when creating the juvenile baseline may have further hindered the freshwater assignment classification in identifying adult sea trout back to their putative regions. With hindsight it may have been more prudent (licences permitting) to sample a larger number of rivers within each of the 10 sub-regions to enable chemical signatures to be obtained which may have been more representative of that given region and which were more likely to be represented in any marine catches of adult fish.

Another factor to be considered in the poor assignment of adults may be the effects of temporal instability in the biogeochemistry of the freshwater baseline. Age estimates of both the adult and juvenile trout were not conducted within the time frame of the present study. However, estimates of the time line for the parr used to create the baseline was 2008-2010 (see section 4.7.4.), with age approximations of adult fish caught during 2010-2012 estimated to infer the period of time spent in freshwater 2008-2010, with the possibility some fish may have resided in freshwater pre-2008 (section 4.7.4.). Results in Chapter 3 and other published studies (Rooker *et al.*, 2001; Gillanders, 2002; Kerr *et al.*, 2007; Walther *et al.*, 2008) have indicated temporal stability between consecutive years is often present. If the assumptions of temporal stability are correct between consecutive years and verification of individual adult trout age confirm the trout were within freshwater between 2008-2010, then the observation with regards to the freshwater

residency period of those adult trout in the years preceding 2008 (*i.e.* 2006-2007) may not be one of the causes for the weak regional signal.

If the freshwater baseline is indeed robust and the problems of temporal stability are unlikely to explain the weak classification of adult sea trout caught in a given marine zone to an adjacent freshwater sub-region, then we may come to the assumption that adult sea trout may be undertaking more extensive migrations than previously thought. Classification using sub-region to determine what percentage number of adult fish are putatively derived from each freshwater sub-region are captured in each marine zone suggests this statement, with extensive migratory patterns observed for adults within the Irish Sea (see Figure 4.21.). Furthermore a possible correlation between extensive movements of adult sea trout and the utilisation of the main sea surface currents within the Irish Sea (Figure 4.22.) observed for the present study further add to this hypothesis.

If we are to assume adult sea trout do undertake more extensive migrations than previously believed more information is required to corroborate the results presented here. The combination of other "natural" tags such as genetic and isotopic analyses with otolith microchemical tags may provide a method for answering this question. It was beyond the scope of this present study to examine the performance of genetic and isotopic natural tags as tools to identify geographical location of origin. However, research has suggested their inclusion in studies to infer movement patterns of fish have been successful (see studies by Walther and Thorrold, 2008; Walther *et al.*, 2008; Perrier *et al.*, 2011; Ramsay *et al.*, 2012) and that a combination of these techniques may improve and provide a better understanding of adult sea trout movement patterns within the Irish Sea.

In conclusion, biogeochemical signatures using Mg, Mn, Sr and Ba obtained from juvenile *Salmo trutta* parr has been shown to be a reliable and consistent tool in inferring natal origin for this study. Additionally the use of a freshwater baseline allowed juvenile parr samples (which were run blind) to be assigned to their natal river with high classification accuracy adding confidence to the freshwater baseline. If temporal stability of the elements used in the creation of the freshwater baseline indicates little variation over two or more years then the poor assignment of migratory adult sea trout to their putative region of origin using their freshwater period of residency may be a result of more extensive migration patterns than previously understood.

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Appendices

Appendix A

Methods for equipment preparation (acid cleansing) and otolith extraction

Equipment preparation

Methods used at the Nuffield Fish Laboratory

Prior to all the fish samples in the storage experiment (Chapter II) and both the temporal study (Chapter III) and regional analysis (Chapter IV) being dissected and the extraction and storage of those otoliths, all instruments used in the removal and storage of the otoliths (*e.g.* plastic tipped forceps, glass beakers, petri dishes, 1.5 ml polypropylene micro-centrifuge tubes and polypropylene wash bottle) were acid washed in 10% Nitric acid (>69% analytical grade HNO_3 Sigma-Aldrich) diluted to 10% with Millipore™ ultra-pure Milli Q water (hereafter referred to as Milli Q; water filtered through an ion exchange cartridge with a value $\geq$ 18.2MΩ cm^{-1} at 25 °C 18 www.millipore.com) for a total of 3 days. The equipment was then triple rinsed in Milli Q and dried for a further 24 hours in a positive flow laminar fume hood and stored in sealed plastic bags prior to use. To prevent the risk of zinc contamination due to aerosolized particles (zinc contained within the glove powder, see Batley 1989, Friel *et al.*, 1996, Dugan *et al.*, 2008) powder-free nitrile gloves (**Fisher**brand) were used during all extraction, handling and storage procedures of the control samples and subsequent handling of the remaining fish for the storage analysis.

Methods used at the B.G.S for equipment (acid) cleansing.

ICP-MS Tube Preparation

To ensure all trace elemental analysis of the bass otoliths conducted at the B.G.S. would not be hindered by the inclusion of contaminate residue within the ICP-MS tubes occurring during manufacture, all ICP-MS tubes were acid washed to a set protocol devised by Dr. S. Chenery (Co-supervisor, B.G.S.). This method of acid washing ICP-MS tubes has been shown to be the best method for removing any contaminates that may be present within the

tubes (Chenery *pers. comm.*, 2008) and has been implemented by Dr. Chenery at the B.G.S. for any future otolith microchemistry analysis using solution-based ICP-MS.

The acid washing method of ICP-MS tubes implemented at the B.G.S. is split into four parts. The first part of the acid washing protocol requires the ICP-MS tubes to be submerged in an acid bath containing 3 litres of an acid wash solution made up from the following acid concentrations; a solution of 1 % Nitric acid (>69% analytical grade HNO_3 Sigma-Aldrich, diluted to 1% with 18.2MΩ ultra-pure water, Milli Q) combined with 0.5% Hydrochloric acid (37 % HCl Sigma-Aldrich, diluted to 0.5 % with 18.2MΩ ultra-pure water, Milli Q). The ICP-MS tubes are then left to soak for a period of 24 hours. To enable the correct concentrations to be calculated and therefore used in the manufacture of the acid bath at larger volumes to contain greater numbers of ICP-MS tubes the dilution equation 1 was used.

Equation 1: $C_i \times V_i = C_f \times V_f$

where C_i is the initial concentration and V_i is the initial volume, with C_f and V_f being the final concentration and volume respectively.

The second part of the acid washing protocols requires the ICP-MS tubes to be emptied of the acid solution of 1 % HNO_3 and 0.5% HCl and then triple rinsed with ultra-pure Milli Q water and left to dry in a positive flow laminar fume hood for 24 hours. The third part of the acid washing protocol involves each of the ICP-MS tubes having a solution of the above acid wash (1 % Nitric acid and 0.5% Hydrochloric acid) decanted into each individual ICP-MS tube (up to the 12 ml mark), with each tube then placed into a tube rack and placed in a fume hood within a clean room (class 1000 B.G.S) and left for a further 24 hours. The fourth and final acid washing protocol requires the ICP-MS tubes to be emptied (making sure the tube is rotated while pouring out the acid to remove any contaminates that may be around the neck of the tubes), triple rinsed in Milli Q water and left to dry for a final 24 hours in a positive flow laminar fume hood.

ICP-MS Tube Preparation at the Nuffield fish laboratory

To reduce the time involved in acid washing and drying the ICP-MS tubes (4 days) at the B.G.S. and maximize the time spent on running samples on the ICP-MS, an assessment of the ICP-MS tube cleaning procedures (using the acid washing protocols set up by Dr. Chenery) was conducted in the Nuffield Fish Laboratory. To assess both the cleaning and

rinsing protocols of the acid washing procedure, two sets of ICP-MS tubes (20 in total) were used. The first set of ICP-MS tubes (n = 10) were pre-washed at the B.G.S. using part one and two of the acid washing protocol (see above), with the second set of ICP-MS tubes (n = 10) having been left un-cleaned and requiring all parts of the acid washing procedure to be implemented. Both sets of pre-washed and un-cleaned tubes were prepared at the Nuffield fish laboratory and then analysed on the inductively coupled plasma mass spectrometer at the B.G.S. to ascertain their purity. The results obtained for both sets of ICP-MS tubes acid washed at the Nuffield fish laboratory and the British Geological Survey after analysis on the Agilent 7500 mass spectrometer indicated the ICP-MS tubes acid washed at the Nuffield fish laboratory were of the high standard required for use in solution based ICP-MS. The statistical tests indicated both sets of tubes were not significantly different (T-test: *P* ranged from 0.153 – 0.923; Appendix A Table 1) and enabled ICP-MS tubes to be prepared in the Nuffield Fish Laboratory ensuring the continued high standard for the analytical procedures required by the B.G.S.

Otolith preparation

Otolith preparation for ICP-MS

After each of the time periods designated for the storage experiment (1, 5, 25 days, 6 and 12 months) were reached, samples for that specific time period were removed from the storage freezer and taken to the analytical laboratory in the Nuffield Fish Laboratory where the process of otolith removal was conducted. Similarly, the samples used for the temporal stability and the spatial variability analysis were conducted in the same laboratory and were subjected to the same extraction protocols set out below. The process of removing the otoliths followed a strict protocol (set up by the present author) not only for the removal of the otoliths but also for the equipment used. Equipment used was first pre-acid washed in 10% Nitric acid (see equipment acid washing procedures in Equipment Preparation) and triple rinsed in Milli Q before each otolith extraction. To facilitate the extraction of the otoliths and to reduce the time the otoliths remained in the head of the fish after thawing, an extraction and wash method was set up as described below. Before each otolith extraction the forceps were first rinsed in 10% HNO_3 then triple rinsed in Milli Q. This procedure was repeated for each fish sample and is made easier by the use of 4 individually marked beakers, one containing the 10% HNO_3 solution and the other 3

containing Milli Q. The now extracted fish otoliths are then placed into a pre-acid-washed petri dish containing Milli Q and cleansed of any remaining tissue using an acid-washed fine bristled nylon brush. The now washed and cleansed otoliths are then placed into a another pre-acid washed petri dish containing three drops of Milli-Q and triple rinsed. Both the left and right sagittal otoliths were then removed from the Milli Q water and placed into separate, correctly labelled acid-washed 1.5ml polypropylene eppendorfs and transported to a positive flow laminar cabinet (BIGNEAT, model OB4HB) in eppendorf racks for a period of 24 hours for drying.

Ultrasonic cleansing

Prior to the otoliths being weighed, and before they were dissolved for analytical analysis using sb- ICP-MS, both the left and right otoliths were subjected to ultrasonic cleansing. This method would allowed any of the remaining adhering tissue trapped within the interstitial spaces (which may have been overlooked when using the nylon brush in Milli Q water) to be removed (see Brophy *et al.*, 2003 for endolymph residue in the interstitial spaces of larval otoliths < 50 μm). To assist in the removing of any remaining adhering tissue using the ultrasonic bath 1 ml of 3% hydrogen peroxide solution (3% H_2O_2 diluted from a concentration of 30% H_2O_2 using Milli Q water) was pipetted into the eppendorfs containing the rinsed otoliths using a Genex Beta 100-1000 μl pipette with an acid washed polypropylene disposable tip and placed into a plastic eppendorf rack and placed into the ultrasonic bath (Grant, model XB22, www.grant.co.uk). The eppendorfs containing the otolith and the 3% solution of H_2O_2 were then subjected to 5 minutes of sonication cleaning. After 5 minutes sonication, the rack containing the eppendorfs was removed and the solution of H_2O_2 and the otolith contained within were emptied into pre-acid washed petri dishes with each otolith subjected to a further triple rinse in Milli Q to dilute and remove any remaining H_2O_2 and then placed into new pre-acid washed correctly labelled polypropylene eppendorfs. The eppendorfs were then placed into a plastic rack and then placed into the positive laminar flow cabinet where they were air dried for a further 24 hours. After the 24 hours in the positive laminar flow cabinet, the 1.5ml polypropylene eppendorfs containing the sagittal otoliths were then capped to prevent any possibility of contaminants entering the eppendorfs during transportation to B.G.S Keyworth and their subsequent insertion in the ICP-MS for elemental analysis.

Appendix A Table 1. Results (using a T/test) for the evaluation of the ICP-MS tube cleaning methods used between the Nuffield fish laboratory and the British Geological Survey.

Element	Tube Preparation	Tube Preparation	Tube *n* =	DF	*T* value	*P* value
Li	Fish Lab	B.G.S	20	17	1.07	0.302
Na	Fish Lab	B.G.S	20	17	0.10	0.920
Mg	Fish Lab	B.G.S	20	17	1.52	0.147
Al	Fish Lab	B.G.S	20	13	0.67	0.514
K	Fish Lab	B.G.S	20	17	0.10	0.923
$Ca^{(42)}$	Fish Lab	B.G.S	20	17	0.93	0.367
$Ca^{(44)}$	Fish Lab	B.G.S	20	17	0.53	0.606
Mn	Fish Lab	B.G.S	20	13	1.35	0.201
Fe	Fish Lab	B.G.S	20	09	1.56	0.153
Cu	Fish Lab	B.G.S	20	16	1.03	0.317
Zn	Fish Lab	B.G.S	20	15	0.59	0.561
As	Fish Lab	B.G.S	20	14	1.26	0.228
Rb	Fish Lab	B.G.S	20	17	0.12	0.907
Sr	Fish Lab	B.G.S	20	17	0.60	0.556
$Cd^{(112)}$	Fish Lab	B.G.S	20	15	0.67	0.513
$Cd^{(114)}$	Fish Lab	B.G.S	20	17	0.43	0.674
Sn	Fish Lab	B.G.S	20	17	0.38	0.710
Ba	Fish Lab	B.G.S	20	17	1.18	0.253
Pb	Fish Lab	B.G.S	20	17	0.21	0.832
U	Fish Lab	B.G.S	20	17	0.47	0.641

* DF = degrees of freedom.

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On-line References

www.millipore.com Milli Q; water filtered through an ion exchange cartridge with a value $\geq$ 18.2MΩ cm^{-1} at 25 °C 18.

Appendix B

Anaesthetics used for dispatching fish at Bluewater fish farm

Anaesthetics used

MS-222 [Tricaine methanesulfonate]: Dissolve 1g of MS-222 and 1g of sodium bicarbonate in 100ml of distilled water. This will create a stock solution of 10mg / ml of solution. The solution was labelled and dated and stored in a dark container to protect it from the light (photo reactive) for a period of no more than three months. 60 ml of MS-222 stock solution was added to 4 litres of sea water (collected from the hatchery containing the sea bass at a temperature ≈25°C) in a plastic bucket into which the sea bass were then placed 5 fish at a time until they were euthanized.

Clove oil: Each ml of clove oil contains approximately 1g of eugenol. To enable the solution to dissipate within the water (eugenol is not completely soluble in water) the clove oil was diluted to 1 part clove oil to 9 parts ethanol (1:10 in 95% ethanol) to create a stock solution of 100 mg / ml. 500ml of the stock solution was then added to 4 litres of sea water and again the same protocols as used for the MS-222 were observed when placing the sea bass into the anaesthetic.

2-Phenoxyethanol: 2-phenoxyethanol was added (at a volume of 7.5 ml) into a plastic bucket already containing 4 litres of sea water collected from the hatchery containing the sea bass at a temperature ≈25°C. The sea bass were then placed into the bucket containing the anaesthetic in groups of 5 and euthanized in turn.

Appendix C

Techniques used to identify and adjust outliers observed in the analytical data (Grubbs and Winsorisation)

Grubbs and Winsorisation analysis

Samples that have been assessed for erroneous data points above and are to remain within the whole data set are then analysed for significance in their distribution from the other remaining concentrations within each element and possible effects for being an outlier using the Grubbs test. The Grubbs test (Grubbs, 1950, 1969) is a statistical test that can be used for detecting statistical outliers within a data set (Hawkins, 1980; Barnett and Lewis, 1994). The assumption of the Grubbs test is to quantify how different the suspected outliers are from the other data points within the sample by using the critical Z value and calculates the difference between that outlier with the mean of the sample set divided by the standard deviation. Therefore, the test indicates where the largest absolute deviation from the whole sample mean (including the outlier) using the components from the whole sample standard deviation (again including the outlier).
The equation is written as follows:

$$G = \frac{\max_{i=1,\ldots,N} \left| Y_i - \bar{Y} \right|}{s}$$

where $\overline{Y}$ represents the sample mean and S represents the standard deviation.

To enable both single and multiple outliers to be assessed for their significance both an online software package (GraphPad Software www.graphpad.com) and a more common statistical package (Minitab) were used to identify possible outliers by calculating the mean and then dividing by the standard deviation. After conducting the first Grubbs test, the outliers identified are either calculated as being significantly different from the sample mean ($P < 0.05$) or not significantly different from the sample mean but are however, identified as a point as being furthest from the other data points ($P > 0.05$). Outliers that were detected using the Grubbs test were then indicated on a scatter plot for each of the seven elements to be used further in the statistical analysis. After applying the Grubbs method of analysis, the now identified outliers may be removed if the researcher considers

there may be a possible external consideration to be taken into account of the sampling protocols or indeed the experimental design. However, another alternative approach to the removal of the outlying data point(s) would be to subject these possible erroneous data point(s) to a process known as Winsorisation (Hawkins, 1980; Sokol and Rohlf, 1995). This method looks at all the data above and below the 5^{th} and 95^{th} percentile (respectively) and sets the numbers which are above and below those parameters (previously identified by the Grubbs test as an outlier) closer to the central observations for that particular data set (Hawkins, 1980; Sokol and Rohlf, 1995). Theoretically, Winsorisation removes the highest and lowest numbers in the data (previously identified by the Grubbs test) and then replaces those numbers with the next highest and lowest adjacent values in the whole data set for that element. This procedure has been classed as a more robust test for outliers in that the identified data points are not completely discarded or omitted and that the test of Winsorisation can be legitimately performed twice, in that both the highest and lowest values identified can be adjusted within that data set (Zar, 2005). The relative efficiency when estimating the means using this method is above 90% when analyzing sample sizes greater than 5 (Sokol and Rohlf, 1995). Outliers that were identified using the above Grubbs test were then subjected to Winsorisation, allowing these previously identified and subsequently corrected data points (using the above Winsorisation method) to be used for further statistical data analysis. Both the Grubbs and Winsorisation methods combined for this study allow the identification and then subsequent re-adjustment of those outliers identified, allowing assurance of the legitimacy of those tests used and their use in subsequent outlier data adjustment for the thesis.

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