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Scales and otoliths as biogeochemical tags of Salmo trutta

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Scales and Otoliths as Biogeochemical Tags of *Salmo trutta* L.

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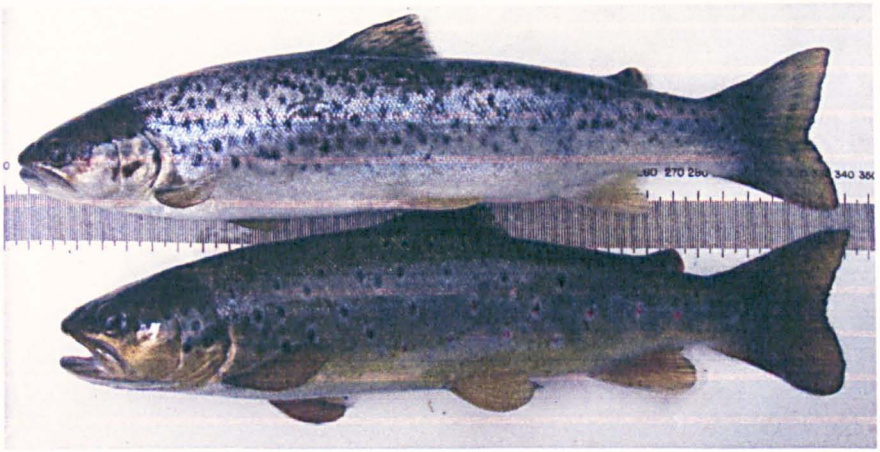

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Abstract

The potential for naturally occurring element concentrations and stable isotope ratios to provide biogeochemical tags of *Salmo trutta,* an andromous salmonid, was examined.

Statistically significant differences were found in the concentrations of Li, Mg, Mn, Sr, Ba and Pb in the hydroxyapatite freshwater growth bands of wild *Salmo trutta* scales collected from 12 catchments draining into the Irish Sea and Celtic Sea. However, only 50% of the fish studied could be correctly classified to their catchment of origin based on element concentrations in scales. The degree to which element concentrations in sea trout scales could be used as a stock discrimination tool of fish in the Irish Sea and Celtic Sea is probably limited.

Sr, Mn, Ba and Mg in recently deposited scale hydroxyapatite and otolith aragonite allowed the classification of *Salmo trutta* to site of origin (n=6) in the Dee river catchment in Wales, U.K., to be determined with 86% and 89% classification accuracy respectively. There appeared to be regional patterning in scale and otolith chemistry in the upper, middle and lower regions of the catchment. Scales might offer a non-lethal biogeochemical tag, comparable in performance to otoliths, but further work needs to examine the degree of post-depositional change in scale hydroxyapatite.

Concentrations of Sr and Mn in scales were correlated with concentrations in stream water, providing a unique opportunity to map high resolution catchment-wide variability in scale element concentrations using British Geological Survey (BGS) stream water chemistry data as a proxy for Sr and Mn in scales, at 792 sites in the Dee catchment (neighbouring sites were >41m apart). The regional patterning in Sr and Mn in scales among sites (n=12) in 3rd order tributaries in the upper, middle and lower regions of the catchment were not present in predicted scale chemistries at sites in 1st and 2nd order tributaries (n=792). Some geographically isolated sites in the Dee catchment might be expected to show similar concentrations of Sr and Mn in scales suggesting that the technique is unlikely to be a useful fisheries research tool in the Dee catchment.

The performance of $\delta^{15}N$, $\delta^{13}C$ and δD in scales and concentrations of Sr, Mn, Ba and Mg in scales and otoliths of *Salmo trutta* as biogeochemical tags of fish at sites (n=6) in the Dee catchment were compared. No significant differences in δD

in scales were detected among the 6 study sites. However, significant differences were detected in $\delta^{15}N$ and $\delta^{13}C$ among sites. The $\delta^{15}N$ and $\delta^{13}C$ values allowed fish to be classified to their site of origin with 93% accuracy. This classification was superior to that achieved when using Sr, Mn, Ba and Mg in the hydroxyapatite of scales from the same fish (which achieved a classification accuracy of 88%). $\delta^{15}N$ and $\delta^{13}C$ could provide biogeochemical tags of *Salmo trutta* but further work needs to examine the degree of spatial variability in $\delta^{15}N$ and $\delta^{13}C$ in *Salmo trutta* scales in the Dee catchment.

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Thesis Contents

Thesis Structure

The overall aim of the thesis is to examine whether calcified structures [specifically scales and otoliths (fish ear bones)] of *Salmo trutta* L. provide biogeochemical tags of populations of the species. The thesis introduction (Chapter 1) provides background information on the ecology of *Salmo trutta* and its population status. The current knowledge deficits regarding *Salmo trutta* ecology are then outlined, with emphasis on those which are impeding the conservation and management of the species. Potential fisheries research methods for addressing these knowledge deficits are then described, with particular emphasis on the use of artificial and natural fish tagging techniques. The use of element concentrations and stable isotope ratios in the calcified structures of fish as biogeochemical tags of fish populations (or stocks*) is then discussed. Background information on the pathways and mechanisms by which elements become associated with fish calcified structures is summarised (supplementary information on the factors affecting the incorporation of elements in fish calcified structures is provided in Appendix B). The thesis objectives are then outlined.

Four data chapters (Chapters 2-5) address the research objectives of the thesis. These have been written in the style of journal manuscripts. Figures, Tables and references can be found at the end of each chapter. While there is some degree of repetition in the methods sections among chapters, this has allowed subtle differences in each of the studies to be discussed. Some supplementary data is provided in Appendices A-E. The findings of the thesis research are summarised in the Discussion chapter (Chapter 6) which includes suggestions for future research.

* The term 'stock' is defined by Cushing (1968) as a sub-group of individuals within the group that is recognised by taxonomists as a species [Cushing, 1968. In: Begg, G.A. & Waldman, J.R. (1999). An holistic approach to fish stock identification. *Fisheries Research*. 43(1-3):35-44]. For the purpose of this thesis, the terms 'population' and 'stock' will be used interchangeably.

Chapter 1

General Introduction

Salmo trutta L.; Distribution and Life History

Salmo trutta belong to the family Salmonidae (Nelson, 2006), which is characterised by fish that spawn in freshwater. The sub-family Salmoninae comprise species which exhibit anadromy. Anadromy describes a life history strategy which involves spawning in freshwater and subsequent migration of fish to the marine environment, usually to feed and mature, after a period of juvenile growth in freshwater. *Salmo trutta* is highly polymorphic, exhibiting life histories strategies ranging from freshwater residency (common name; brown trout) to periods of time spent in estuarine (common name; slob trout) and marine environments (common name; sea trout). The highly variable life-history strategies exhibited by the species have caused considerable debate regarding its taxonomic classification. *Salmo trutta* was originally described by Linnaeus in 1758 as three separate species; *Salmo fario* (stream trout), *Salmo trutta* (river trout) and *Salmo eriox* (sea trout) (Elliott, 1994). Current classification recognises that despite differences in the morphology and life-history strategies of fish among these groups, they inter-breed freely and should be considered as one species (Elliott, 1994).

The northern limits of the species native geographical range span from North West Russia, to northern Scandinavia and to Iceland. The species is found throughout Europe and on the North West tip of Africa (Elliott, 1989). The native anadromous form of *Salmo trutta* L. (sea trout) is only found on the West coast of Europe and in the Black Sea and Caspian Sea (Elliott, 1989). *Salmo trutta* has been introduced to, and become established in, at least 24 countries including New Zealand, Sri Lanka, Canada, the U.S.A. and several countries in Africa, Asia, South America and the Middle East.

In the U.K., *Salmo trutta* tend to spawn in freshwater in autumn and winter, during which time eggs are buried in gravel redds in freshwater streams/rivers.

Spawning times can be earlier at higher altitudes and latitudes due to lower temperatures (reviewed in Klemetsen *et al.*, 2003). Eggs subsequently hatch during the following spring (reviewed in Klemetsen *et al.*, 2003). After a period of one to three years (usually two years) of juvenile growth in freshwater, *Salmo trutta* either mature as freshwater-resident brown trout or migrate towards the marine environment to mature as sea trout (Jonsson, 1989). *Salmo trutta* migration tends to occur during spring. The extent of migration is highly variable and has been described as a continuum from relatively small scale movements within freshwater (distances <100m) (Milner *et al.*, 1979) to migrations to lacustrine, estuarine and marine environments (Milner *et al.*, 2003). While the downstream movement of juvenile *Salmo trutta* has been associated with environmental triggers such as an increase in water discharge (Arawomo, 1980; Aarestrup *et al.*, 2002) and stream water temperatures (Arawomo, 1980), the extent of fish migration is thought ultimately to depend on the life history strategy which will maximise individual fitness and may be influenced by genetic factors (reviewed in Jonsson & Jonsson, 1993). Faster-growing individuals are thought to migrate more readily and at a smaller body size when compared with slower-growing individuals (Forseth *et al.*, 1999). Migrating salmonids have been found to have higher metabolic demands compared with freshwater-resident fish of the same species (Morinville & Rasmussen, 2003) and this suggests that fish are 'pushed' along a continuum of increasing food availability until their metabolic demands are met (Cucherousset *et al.*, 2005). The size of migratory fish increases with proximity to the sea, corresponding to an improvement in feeding opportunities (Cucherousset *et al.*, 2005) and a shift to larger prey items on entry to the marine environment (Morinville & Rasmussen, 2006).

During migration, fish undergo 'smoltification', a process involving morphological and physiological changes by which they pre-adapt to inhabiting a saline environment (Tanguy *et al.*, 1994). The spectrum of prey types available to anadromous fish in the marine environment is broader when compared with freshwater (Morinville & Rasmussen, 2006). Coupled with larger prey sizes, this can reduce the costs associated with foraging and indicates that rapid growth rates can be maintained in the marine environment (Morinville & Rasmussen, 2006). Adopting a migratory life history strategy is thought to be a trade-off between improved feeding opportunities (which can result in an increase in size-at-age and fecundity) and an increased risk of predation (Jonsson & Gravem, 1985; reviewed in: Jonsson & Jonsson, 1993). Sea trout

generally mature later than their freshwater-resident counterparts which suggests that there might also be a trade-off (in terms of reproductive fitness) between adopting a freshwater-resident life history strategy (which can result in early maturation at a smaller size) and a migratory life history strategy (which can result in delayed maturation at a larger body size with increased fecundity) (reviewed in: Jonsson & Jonsson, 1993). Reproductive fitness in females increases with size because females compete for optimal spawning sites and because egg production increases with size (reviewed in Jonsson & Jonsson, 1993; reviewed in Klemetsen *et al.*, 2003). In contrast, there are two size classes which can maximise fitness in males. Larger males have a direct competitive advantage during spawning but smaller males can maintain reproductive fitness by adopting precocious mating techniques (Cucherousset *et al.*, 2005). The importance for females of achieving a large size may account in part for the higher proportion of females compared with males which migrate to the marine environment for enhanced feeding/growth opportunities (reviewed in Jonsson & Jonsson, 1993).

During marine residency, sea trout are thought to feed in coastal environments (Berg & Berg, 1987; Jonsson, 1989) and may reside at sea for the summer only or for several years prior to undertaking annual trips to their natal river catchment and tributary to spawn (reviewed in: Klemetsen *et al.*, 2003). Depending on survival rates, sea trout in Britain may spawn eleven times or more (Harris & Milner, 2006). Tagging data suggest that the proportion of sea trout that return specifically to their natal tributary is high (Sambrook, 1983; Le Cren, 1985).

The *Salmo trutta*; Population Declines of a Socio-economically Important Species

Sea trout are an important socio-economic resource in British waters and provide valuable sport and commercial fisheries (Elliott, 1989; O'Reilly & Mawle, 2006). In England and Wales alone salmonid net fisheries can generate a gross annual income of £160,000 (O'Reilly & Mawle, 2006). While it is difficult to dissociate the value contributed by sea trout from that contributed by Atlantic salmon (*Salmo salar*), it has been suggested that the relative value of the two species is similar (O'Reilly & Mawle, 2006). Rod fishing for both sea trout and salmon is a popular sport in the region with

an estimated total value to fishery owners of over £100 million (O'Reilly & Mawle, 2006).

Since the late 1980s, populations of sea trout (*Salmo trutta*), have suffered severe declines in the West of Ireland and since the early 1990s in some areas of Scotland (Dawson, 1998; MacKenzie *et al.*, 1998; Harris & Milner, 2006). A recent analysis of sea trout population monitoring data for the West of Ireland indicates that populations have not yet recovered to pre-collapse levels (Gargan *et al.*, 2006). The stock collapse in the 1980s and 1990s highlighted the vulnerability of sea trout populations in England and Wales (Harris & Milner, 2006) and elsewhere in the species native range. Given that some sea trout populations have declined markedly, the commercial value of sea trout fisheries is potentially at risk (Youngson *et al.*, 2003) in British waters.

While the reasons for the species population declines are unclear, there are a number of factors that are thought to effect the survival rates of sea trout in the marine environment, including drift net fishing and the potential for cross-infection of sea lice (particularly sea lice species *Lepeophtheirus salmonis* and *Caligus elongatus*) between farmed populations of salmonids and wild populations of sea trout (MacKenzie *et al.*, 1998). Sea lice infestations can cause the premature return of sea trout to freshwater (Birkeland & Jakobsen, 1997) and might cause mortality, depending on the severity of the infestation (Bjørn *et al.*, 2001). However, there have been declines in sea trout numbers in areas without fish farms and this suggests that other factors may be involved (MacKenzie *et al.*, 1998). Further research is required on the species population dynamics in the marine environment in order to determine where and how mortality occurs (Potter & Crozier, 2000). The degree to which conditions in freshwater might contribute to population declines of the species is unclear. During juvenile life stages, siltation of spawning gravels [which can have a deleterious effect on the survival of eggs (Acornley & Sear, 1999)] and alterations of natural flow regimes and physical habitats might limit the productivity of *Salmo trutta* populations (Armstrong & Nislow, 2006). During adult life stages, water pollution (Kemp & Spotila, 1997), altered flow regimes and physical barriers to the upstream migration of *Salmo trutta* during the spawning run (Gosset & Labonne, 2006) could also be expected to limit productivity.

Salmo trutta conservation and management

Protecting and improving existing population levels of sea trout has become a primary aim for the management of *Salmo trutta* (Youngson *et al.*, 2003). Current management practices are often designed to restore populations of the species in native rivers and can include the enhancement of wild populations by stocking rivers with hatchery-reared *Salmo trutta* (Youngson *et al.*, 2003). Improving spawning habitats, habitat connectivity in freshwater and reducing the exploitation of the species in both marine and freshwater are also likely to be beneficial for the species and can be considered more favourable than stocking.

Successful conservation and management of fish populations requires a thorough understanding of the ecology of fish species, in particular their distribution and inter-mixing of populations. More specifically, it is dependent on understanding the importance of habitats at different life stages, habitat connectivity (particularly relevant in the management of anadromous species), the chronology of life history events and their relationship with improving fitness (Feyrer *et al.*, 2007).

A number of methods have been developed for researching the habitat use and migration patterns of fish and for discriminating between populations or stocks of fish in both marine and freshwater environments. To date, the methodologies employed in identifying fish have involved the use of 'tags', unique to individuals or populations. Tags can be grouped into those which are 'artificial' (i.e. applied to the fish by the researcher), and those which are 'natural' (i.e. naturally occurring in individuals or populations).

Artificial tags

Coded-wire tags (Courtney *et al.*, 2000), radio tags [such as the Passive Integrated Transponder (PIT)], microchips and acoustic tags are examples of artificial tags which have been used to examine migrations of fish and inter-mixing of stocks (Begg & Waldman, 1999). While the use of artificial tags has improved our understanding of the ecology of fish species, including *Salmo trutta* (Cucherousset *et al.*, 2005), there are limitations with this technique. For instance, a large number of tags need to be deployed/recovered in order to generate meaningful population data (Gillanders,

2005b). Due to the monetary and labour costs associated with achieving this, the application of artificial tags has generally been restricted to studying fish over relatively small geographical and temporal scales. Unfortunately, using artificial tags on juvenile fish can cause handling effects or mortality and so the technique can rarely be used to study the entire life history of individual fish (Gillanders & Kingsford 2000; Elsdon & Gillanders, 2005). Alternative artificial tags can involve fin clipping or inducing a change in the element composition of sequentially deposited growth rings in calcified structures in fish [e.g. vertebrae and otoliths (fish ear bones)]. Exposing fish to high concentrations of tetracycline (Lorson & Mudrak, 1987), strontium (Schroder *et al.*, 1995) or lanthanide elements (rare earth elements) (Ennevor & Beames, 1993) has provided a chemical 'tag' in fish structures which can be stable throughout the lifetime of individual fish, and which can be applied during larval life stages.

Natural tags

Natural tags exploit naturally occurring differences in, for example, morphometric (body, otolith and scale shape), meristic (e.g. number of gill rakers and fin rays) (Casselman *et al.*, 1981; Haas-Castro *et al.*, 2006) and genetic (Bembo *et al.*, 1996) characteristics among populations of fish (reviewed in Begg & Waldman, 1999). Differences in the bacterial (Smith *et al.*, 2007; Wilson *et al.*, 2008) and parasitic assemblages (Lester *et al.*, 1988) of fish have also been used to discriminate between populations originating from different geographical locations. The primary advantage of using natural tags as opposed to artificial tags is that they do not have to be applied to the fish by the researcher (Begg & Waldman, 1999; Feyrer *et al.*, 2007; Elsdon *et al.*, 2008).

Variations in naturally occurring stable isotope ratios and element concentrations in the calcified structures of fish (e.g. otoliths and scales; herein described as 'structures'), have also provided tags of fish populations (Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005; Comyns *et al.*, 2008). To date, variability in fish structure chemistry has been found in fish within and among riverine (Wells *et al.*, 2003a), lacustrine (Bronte *et al.*, 1996), estuarine (Swearer *et al.*, 2003), coastal (Brown, 2006) and open ocean (Rooker *et al.*, 2001; Brophy *et al.*, 2003)

environments. The majority of research in this area has focused on applying the technique to study fish inhabiting estuarine/coastal environments and the technique has proved useful for discriminating between stocks and revealing migratory patterns within/among these habitats (Swearer *et al.*, 2003; Gillanders, 2005b). The technique has also proved useful in distinguishing fish originating from different locations in freshwater (Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007). Chemical elements are incorporated into calcified structures of fish during structure formation and can reflect element concentrations and stable isotope ratios in the local environment at the time of formation (this is addressed in further detail below). As fish calcified structures grow incrementally, forming discernable growth rings, they can offer a chronological record of the environmental chemistry to which a fish has been exposed (Campana & Thorrold, 2001; Pontual & Geffen, 2002), allowing the movements of fish between habitats with differing chemistry to be determined retrospectively (Muhlfeld *et al.*, 2005).

In some cases, elemental tags have been used to identify natal origins of fish at a finer geographical resolution than achieved using other tagging techniques (Bronte *et al.*, 1996; Campana *et al.*, 1999). For instance, Campana *et al.* (1999) found that otolith element concentration data could be used as a natural tag to reveal the relative contribution of stocks of cod (*Gadus morhua*) from different spawning grounds, to overwintering grounds of the species in the Gulf of St Lawrence. This level of discrimination had not been achievable when using artificial tagging or genetic techniques (Campana *et al.*, 1999). Similarly, Bronte *et al.* (1996) distinguished between lake herring (*Coregonus artedi*) from two regions of Lake Superior where no genetic distinctions between fish in the two regions could be made.

Fish structure chemistry as a fisheries research tool

Some of the first studies examining fish structure chemistry as a fisheries research tool took place in the 1950s (Schroder *et al.*, 1995) and since then there have been a number of studies which have explored the use of chemically marking fish calcified structures to provide an artificial tag [e.g. by exposing fish to high levels of strontium (Ophel & Judd 1968; Yamada *et al.*, 1979; Schroder *et al.*, 1995)]. Ennevor & Beams (1993) found that lanthanide elements (rare earth elements) added to ambient water

were absorbed by juvenile coho salmon (*Oncorhynchus kisutch*) and deposited at detectable levels in vertebrae, otoliths and scales, thus providing an artificial tag. Jones *et al.* (1999) artificially marked the otoliths of developing embryos of a damselfish (*Pomacentrus amboinensis*) which enabled the recruitment of 'tagged' fish to their natal reef environment to be determined (Jones *et al.*, 1999).

The exploitation of naturally occurring stable isotope ratios and element concentrations in fish structures as an aid to discriminating between stocks of fish has been an exciting development in fisheries research. The concentrations of some elements in fish structures can be proportional to their ambient concentrations in the surrounding water. Generally speaking, ambient Sr is more prevalent in saline water when compared with freshwater and this difference can be reflected in the concentrations of this element in fish structures. Bagenal and co-authors (1973) conducted one of the first studies to use Sr:Ca concentrations in scales to distinguish between freshwater and marine contingents of an anadromous salmonid (*Salmo trutta*). The use of fish structure chemistry to determine the chronological migration histories of fish across salinity gradients (e.g. among freshwater, estuarine and marine environments) based on Sr:Ca concentrations in the growth bands of fish structures has continued to provide a useful fisheries research tool (Secor & Piccoli, 1996; Secor & Rooker, 2000; Zimmerman & Reeves 2000; Esldon & Gillanders, 2006a; reviewed in Gillanders, 2005a). For instance, Arai and Miyazaki (2001) used Sr:Ca in otolith growth bands to determine the periodicity and duration of migrations of individual semi-anadromous Russian Sturgeon (*Acipenser guldenstadti*) between river catchments and the Caspian sea (Arai & Miyazaki, 2001). In recent years ambient concentrations of Ba in freshwater and marine habitats have also been used to retrospectively infer movements of migratory fish between these habitats (Elsdon & Gillanders, 2003a). Ba is more bioavailable in freshwater when compared with marine water (Elsdon & Gillanders, 2005). It originates in terrestrial systems and is transported into freshwater (Elsdon & Gillanders, 2005). Elsdon and Gillanders demonstrated that otolith Ba:Ca in the otoliths of wild fish was related to ambient Ba:Ca concentrations in water and this enabled the movement patterns of black bream (*Acanthopagrus butcheri*) to be distinguished between marine and freshwater habitats (Elsdon & Gillanders, 2005). Secor and co-authors (1995 & 2001) exploited the positive linear relationship between salinity and concentrations of Sr in otoliths of Hudson River striped bass (*Morone saxatilis*) to determine coastal, estuarine and

riverine contingents of the species in combination with data on Ba, Na, Mg, K and Mn in otoliths.

In recent years, the majority of studies utilising elemental tags have focused on fish structure chemistry as a stock discrimination tool in estuarine (e.g. Swearer *et al.*, 2003) and coastal (Comyns *et al.*, 2008) environments. Some studies have also been carried out in open ocean environments (e.g. Brophy *et al.*, 2003; Shuford *et al.*, 2007). The technique has proved particularly successful in discriminating the estuary of origin of fish (Thorrold *et al.*, 1998b; Gillanders & Kingsford, 2000), the coastal origins of fish (Comyns *et al.*, 2008; Schaffler *et al.*, 2009), estimating the relative importance of nursery areas in contributing fish to the adult population (Gillanders & Kingsford, 1996; Chittaro *et al.*, 2009) and in determining the degree of natal homing of fish (Thorrold *et al.*, 2001). The technique has also proved useful in discriminating between populations of fish inhabiting estuarine and coastal environments (Brown, 2006). While in some cases the method has offered a relatively poor stock discrimination tool (Kalish *et al.*, 1996; Stransky *et al.*, 2005), many of the studies have generated encouraging results. For instance, Wells and co-authors (2000b) found that concentrations of Mg, Mn, Ba and Sr in the scales of juvenile weakfish (*Cynoscion regalis*) differed among nursery estuaries, indicating that the technique might be useful for retrospectively elucidating the estuary of origin of adult weakfish during pelagic life history stages. The study examined the scales of weakfish from a sample from five estuaries within the species geographical range and found that >65% of fish (from the same year class) could be classified to their estuary of origin. Other studies have also found significant differences in element concentrations in fish structures between estuaries, in particular Mn, Sr and Ba (Swearer *et al.*, 2003). Swearer *et al.* (2003) reported that there appeared to be some degree of regional patterning in otolith chemistry, based on the finding that mis-classified fish tended to be classified to estuaries neighbouring the natal estuary of origin. To date, the technique has been applied at a range of spatial scales and has distinguished fish between sites which are separated by 100s of kilometres (Thorrold *et al.*, 1998b) and more recently at much smaller spatial scales [e.g. sites separated by 15km (Dorval *et al.*, 2005)]. In summary, natural tags have shown promise as a tool for identifying the relative contribution of juveniles from estuaries/coastal regions to mixed stocks of species during pelagic life stages (Campana *et al.*, 1999) in addition to determining connectivity between habitats (Campana *et al.*, 1999; Fodrie & Herzka, 2008).

Fish structure chemistry has also shown promise as a fisheries research tool in freshwater environments. A number of studies have shown that the chemistry of otoliths of anadromous fish can differ among natal catchments (Thorrold *et al.*, 1998a; Veinott, & Porter, 2005; Walther & Thorrold, 2008). Coillie and Rousseau (1974) reported significant differences in a suite of element concentrations in whole scales of white sucker (*Catostomus commersoni*) between two rivers, one of which suffered anthropogenic pollution. Veinott & Porter (2005) found that concentrations of Mg, Mn, Sr and Ba in the otoliths of juvenile Altantic salmon (*Salmo salar*) differed significantly among fish from three rivers in Newfoundland and this enabled the river of origin to be determined with >84% accuracy. Similarly, juvenile splittail (*Pogonichthys macrolepidotus*) from four rivers draining into the San Fransisco Estuary in California could be distinguished with 71% accuracy, based on concentrations of Sr:Ca and ^{87}Sr:^{86}Sr in otoliths (Feyrer *et al.*, 2007). More recently, Walther and Thorrold (2008) carried out a study which predicted otolith chemistry of American shad (*Alosa sapidissima*) among 20 natal catchments throughout the species native range on the east coast of America (>2700km of coastline) and distinguished the catchment of origin with 93% accuracy. While the application of biogeochemical tags to determine freshwater origins of fish is still in its infancy, it is likely to continue to generate interest as a fisheries research tool (Gillanders, 2005a).

An exciting application of fish structure chemistry in fisheries research could be to elucidate the natal origins of fish within river catchments. Identifying the natal origin of fish to areas within a catchment would provide a way of determining the productivity of spawning sites contributing migratory fish to adult stocks. To date, the findings suggest that biogeochemical tags show promise as a stock discrimination tool in freshwater. Wells and co-authors (2003a) conducted a study in three streams in the Coeur d'Alene River system in the upper Columbia River Basin in which they compared the Mg:Ca, Mn:Ca, Sr:Ca, and Ba:Ca ratios in the local water with concentrations of these elements in otoliths and scales of 1 year old cutthroat trout (*Oncorhyncus clarki lewisi*) (fish samples were collected during one summer). The relationship between element:Ca in scales and streamwater was used to predict Mg:Ca, Sr:Ca and Ba:Ca in scales in 30 streams within the study region (Wells *et al.*, 2003a). There appeared to be little regional patterning in structure chemistry in the study region and some sites that were geographically isolated appeared to share similar scale chemistries. However, a subsequent study on the same species in the upper Flat Head

River (a neighbouring river system which is also part of the Columbia River Basin), found regional patterning in scale chemistry. Individual fish could be correctly classified to the 3 drainages in the study area with 82% accuracy and then further classified to streams within each drainage with 88% accuracy based on concentrations of Mg:Ca, Mn:Ca, Ba:Ca and Pb:Ca in scales (Muhlfeld *et al.*, 2005). Differences in element:Ca ratios have also been found between fish from different sites within a lake system (Bronte *et al.*, 1996).

The use of fish structure chemistry might also be useful for determining movements of fish within freshwater. Palace and co-authors (2007) exploited river runoff from a coalmine to determine tributary residence of rainbow trout (*Oncorhyncus mykiss*) in a river catchment based on Se in otolith growth bands.

One of the first steps in determining the suitability of fish structure chemistry as a stock discrimination tool is to determine if fish residing in different areas do in fact have different element 'signatures' (Gillanders, 2005b). To date, the majority of studies carried out in freshwater have been conducted in large catchments (~5,000-100,000skm^2) and have sampled fish structure chemistry at a selection of sites within a study region (Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007; Adey, 2007). While this 'site-based' sampling design gives an indication of the degree to which fish structure chemistry might vary within a watershed, baseline element concentrations in fish structures should ideally be determined for all source groups contributing fish to the mixed stock (Gillanders, 2005b). Occasionally there appears to be some regional patterning in the fish structure chemistry among sites in the study area (e.g. Bronte *et al.*, 1996; Muhlfeld *et al.*, 2005). However, failure to account for possible fine scale variability in baseline chemical signatures, could result in the erroneous classification of fish to site of origin and/or overestimating the accuracy with which spatial movement patterns of fish can be determined (Campana *et al.*, 2000; Gillanders, 2005b; Elsdon *et al.*, 2008). The degree to which fish structure chemistry might vary on small spatial scales within a watershed is yet to be determined.

Naturally occurring variability in stable isotope ratios have also provided natural tags of fish in both marine (Clark *et al.*, 2009) and freshwater (Kennedy *et al.*, 1997; Kennedy *et al.*, 2005) environments. Kennedy and co-authors (2005) found natural variations in $\delta^{15}N$ and $\delta^{13}C$ scales and ^{87}Sr:^{86}Sr in otoliths of Atlantic salmon (*Salmo salar*) among 12 tributaries of the Connecticut River, USA. $\delta^{15}N$ and $\delta^{13}C$

alone could discriminate the site of origin of fish with 73% accuracy. Schmetterling and Dawson (2002) also found that juvenile westslope cutthroat trout (*Oncorhynchus clarki lewisi*) caught in different tributaries within a catchment could be differentiated based on $\delta^{13}C$ and $\delta^{15}N$ in scales (Schmetterling & Dawson, 2002). $\delta^{15}N$ in fish scales have been related to land use (Harrington *et al.*, 1998; Kennedy *et al.*, 2005) which probably reflects variability in the application of fertilisers in the catchment (Harrington *et al.*, 1998). Kennedy and co-authors (1997 & 2000) have carried out a number of studies which have found that $^{87}Sr:^{86}Sr$ in otoliths have provided natural tags of fish in freshwater. Barnett-Johnson *et al.* (2008) carried out one of the first studies in freshwater to quantify the relationship between geology and $^{87}Sr:^{86}Sr$ in streamwater and otoliths of Chinook salmon (*Oncorhynchus tshawytscha*) in the California Central Valley.

Combining naturally occurring element concentrations and stable isotope ratios in fish structures can enhance the accuracy with which fish origins can be determined (Thorrold *et al.*, 1998b). While studies have rarely compared the relative contribution of element concentrations and stable isotope ratios in discriminating stocks of fish, Thorrold and co-authors (1998b) found that analysing $\delta^{13}C$ and $\delta^{18}O$ in combination with Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca in otoliths increased the accuracy of assigning juvenile weakfish (*Cynoscion regalis*) to natal estuarine areas. Other authors have also found that stable isotope ratios ($\delta^{13}C$ and $\delta^{18}O$) can enhance the discrimination of stocks of fish in coastal environments when combined with element concentrations (e.g. Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca) (Clark *et al.*, 2009). While the relative contribution of different stable isotope ratios (e.g. $\delta^{15}N$, $\delta^{13}C$ and $^{87}Sr:^{86}Sr$) to discriminating fish among locations in freshwater has been examined (Kennedy *et al.*, 2005), quantifying the degree to which element *concentrations* compared with stable isotope ratios might assist in discriminating between fish among freshwater locations is yet to be determined.

Processes by which elements are incorporated into fish structures

While the incorporation of elemental 'impurities' into calcified structures has proven to offer a useful tool in fisheries stock discrimination studies, the sources of elements and the pathways and mechanisms by which they can become associated with the

calcified structures of fish is still being understood. A number of studies have tried to address this knowledge deficit. Evidence suggests that the process is dependent on the element in question, and can be influenced by factors such as fish phylogeny, ontogeny, physiology [e.g. Kalish (1992) found some evidence that an increase in stress corresponded to an increase in Sr:Ca in otoliths], age, growth rate, diet and chemical composition of the structure [(e.g. hydroxyapatite (scales and vertebrae) or aragonite (otoliths)] (Fowler *et al.*, 1995a; Fowler *et al.*, 1995b; Clarke *et al.*, 2007; Campana, 1999). While some tentative generalisations can be made, inconsistent results between some of the studies that have examined the relative influence of these factors on element incorporation, suggest that a complex interaction between several factors can be involved (Fowler *et al.*, 1995b) and it can be difficult to isolate the relative importance of those factors that are responsible for causing changes in element concentrations in fish structures (e.g. Kalish, 1992; Fowler *et al.*, 1995b).

The degree to which element concentrations in fish structures reflect element concentrations in the local aquatic environment appears to vary depending on the species in question and the environmental conditions. Elements such as Sr and Ba in fish structures are often found to vary in concentration in response to changes in ambient concentrations in water (Wells *et al.*, 2000a; Muhlfeld *et al.*, 2005), whereas other elements such as N, K, Cl, Zn and Cu are less likely to reflect concentrations in the local environment because they are physiologically regulated (Rooker *et al.*, 2001; Miller *et al.*, 2006). Evidence suggests that elements can be absorbed through epithelia cells in the intestine and the gills (Guerreiro, 2002). There are a limited number of studies which have examined the degree to which diet and water contribute to the elements incorporated into fish structures. Studies have found that over 80% of Ca (Simkiss, 1974), Sr and Ba (Walther & Thorrold, 2006) in otoliths can be derived from water with the remaining proportion attributable to dietary sources (Walther & Thorrold, 2006). Elsdon *et al.*, (2008) suggested that while some elements might represent primarily dietary sources (e.g. S) other elements might be derived mainly from water (e.g. Sr). Limburg (1995) found that altering the diet of freshwater American shad (*Alosa sapidissima*) to a diet of marine fish caused an increase in the Sr:Ca in otoliths, suggesting that diet can be a contributing source of elements (Limburg, 1995). Marine dwelling fish inhabit a hypertonic medium which induces water loss. In order to maintain fluid levels, marine fish absorb more water through the intestine than fish which inhabit freshwater. This might mean that the main pathway

through which marine fish absorb ions is via the intestinal epithelia. In contrast the main pathway for ion uptake in freshwater fish is thought to be via the gill epithelia (Guerreiro, 2002). The findings to date indicate that elements are derived primarily through gills in freshwater fish (Milton & Chenery, 2001; Brown & Severin, 2009) and in some marine fish (Walther & Thorrold, 2006; Lin *et al.*, 2006) although for marine species the relative importance of water and dietary sources is less clear (Brown & Severin, 2009). For instance, Swearer *et al.* (2003) found differences in element concentrations among five species of estuarine fish inhabiting the same geographical area and attributed this to potential variation in diet between species. However, the relative contribution of water and diet to element concentrations was not quantified in this study (Swearer *et al.*, 2003).

The relative importance of water and diet as a source of elements is probably dependant on the element in question and the environment that the species inhabits. Once absorbed, elements are transported to fish calcified structures via the bloodstream. The following section describes the structure of scales and otoliths and the mechanisms by which elements become incorporated into each of these structures. Appendix A contains an overview of the methods for determining element concentrations and stable isotope ratios in fish structures.

Otoliths

The function of fish otoliths is thought to relate to orientation and hearing (reviewed in Campana, 2005). There are three pairs of otoliths in teleost fish (sagitta, lapilli and asterisci). The sagittal otoliths are typically used in biogeochemical tagging studies and are composed of calcium carbonate ($CaCo_3$) primarily in the form of aragonite (as opposed to calcite or vaterite) in a non-collagenous organic matrix (Campana, 1999). Some vaterite can be formed in sagittal otoliths and the degree to which elements are incorporated into calcite and vaterite can vary (Gauldie 1996; Brown & Severin, 1999). For instance, Brown & Severin (1999) found that vaterite was deficient in some elements (e.g. Sr, N and K) relative to aragonite (Brown & Severin, 1999). While vaterite is often detectable by eye (because it can be glassy in appearance in comparison to aragonite which can appear opaque) (Tomás *et al.*, 2004), some smaller and less visible intrusions might also be present (Brown & Severin, 1999). Otolith

growth occurs through differential deposition of calcium carbonate (which crystallises out of the endolymph fluid), and deposition of protein over a 24hr period (Campana & Neilson, 1985). As with scale growth, otolith growth is proportional to fish growth and it is possible to identify annual, seasonal and even daily growth bands/increments (Campana & Thorrold, 2001) (Fig. 1 and 2). The influence of environmental factors, such as temperature, on otolith formation can even cause sub-daily increments to occur (Campana & Neilson, 1985). Seasonal growth patterns are usually discernable under a dissecting microscope, appearing as opaque and translucent bands (Toole *et al.*, 1993). Under transmitted light the opaque zone appears dark and the translucent zone appears light (Wright *et al.*, 2002). The opaque bands tend to be protein-rich whereas the translucent bands tend to be protein-poor material (Toole *et al.*, 1993). The difference in the appearance (opaque and translucent) of seasonal bands can be caused by a number of factors, such as changes in the ratio of calcium carbonate to protein matrix and the thickness and size of aragonite crystals (Wright *et al.*, 2002).

In comparison to other fish calcified structures, otoliths have continued to receive the most attention from fisheries biologists in biogeochemical tagging studies (Campana & Thorrold, 2001) for several reasons. Otoliths start forming prior to hatching and grow continuously throughout the entire lifetime of the fish (Campana & Thorrold, 2001; Campana & Neilson, 1985). In contrast, scales form several days after hatching (Parrott, 1934). Otoliths grow continuously, even during periods of food deprivation (Campana & Neilson, 1985) and can exhibit growth increments over small temporal scales (Campana & Thorrold, 2001), whereas other structures provide poorer chronological resolutions. Although daily growth bands have been identified in scales in some species, this has been restricted to the hydroxyapatite deposited during juvenile life stages (Campana & Neilson, 1985) and usually only seasonal growth bands are discernable. In contrast to scales and bones, otoliths are generally thought to be relatively inert after crystallisation (Campana & Thorrold, 2001). However, there is some evidence that Ca in otoliths might be resorped during times of stress (Mugiya & Uchimura, 1989). Wright and co-authors (1992) found that inducing hypocalcaemia in juvenile Atlantic salmon (*Salmo salar*) caused a short-term loss of calcium from otoliths (Wright *et al.*, 1992). A particular disadvantage of using otoliths as biogeochemical tags is that their collection necessitates the killing of fish which is particularly undesirable when studying rare species (Clarke *et al.*, 2007) and may reduce the market value of commercial catches (Gillanders, 2001).

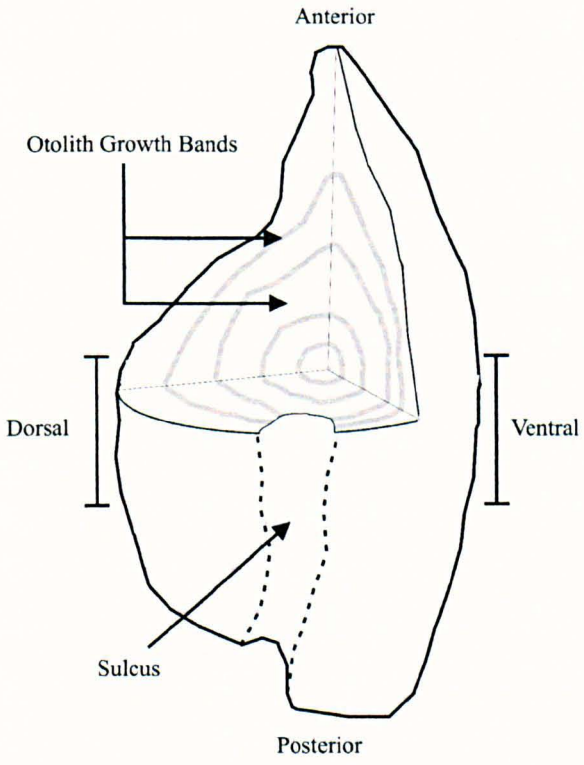

Figure 1. Diagram of a *Salmo trutta* otolith. The anterior, posterior, dorsal and ventral regions of the otolith are labelled according to the otolith diagram for sparidae snapper (*Pagrus auratus*) in Hamer & Jenkins (2007).

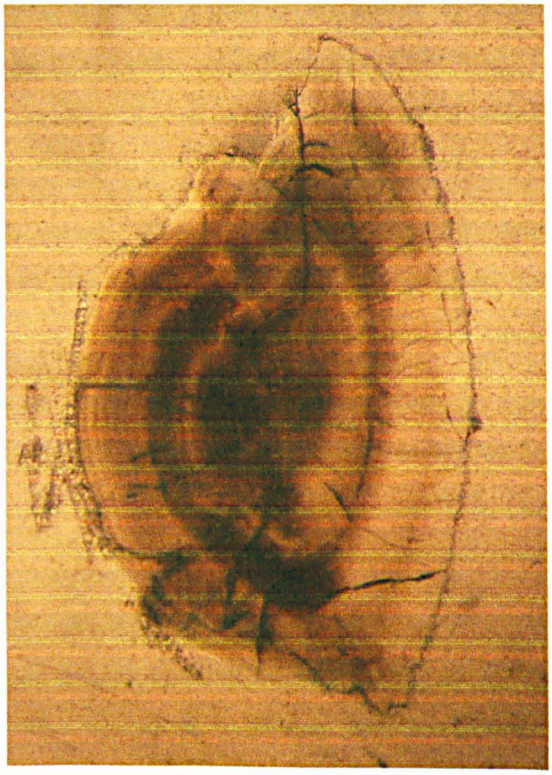

Figure 2. Image of a sectioned freshwater-resident *Salmo trutta* otolith mounted in resin (Fig. 1). Image captured using a camera mounted on a binocular microscope.

Scales

Scales provide protection for the body in addition to performing hydrodynamic functions (Meunier, 2002). Soft-rayed teleost fish such as *Salmo trutta* have cycloid scales, characterised by an outer edge which appears smooth and rounded in planar view, as opposed to spiny-rayed teleost fish which have ctenoid scales that have an outer edge which is toothed or comb-like in appearance (Meunier, 2002). Scales grow incrementally throughout the lifetime of the fish, in proportion to fish growth (Elliott & Chambers, 1996). A teleost scale is composed of two components; a proximal basal plate (also known as the fibrillary plate) which lies against the fish and an external layer (also known as the osseous layer) which forms the outer side of the scale (Fig. 3 and 4). The two components of the scale are very different in their structure and patterns of growth. The basal plate consists of an organic layer of collagen fibres which are arranged vertically within horizontal sheet-like layers that cover the entire underside of the scale (Onozato & Watabe, 1979) (Fig. 4 and 5). Successive collagen layers extend beyond the outer edge of the previous collagen layer, increasing the surface area of the scale as the fish grows (Hutchinson & Trueman, 2006). The basal plate is therefore at its thickest in the central region of the scale (Fig. 4) (Hutchinson & Trueman, 2006). The osseous layer is composed of a calcium phosphate matrix (Hutchinson & Trueman, 2006), similar in composition to the mineral hydroxyapatite [$Ca_5(PO_4)_3(OH)$] (Flem *et al.*, 2005). The growth of the osseous layer occurs by incremental additional of material to the peripheral edge of the scale in the form of circular ridges (Schönbörner *et al.*, 1979). The osseous layer is usually the same thickness irrespective of location on the scale (Fig. 4 and 5) (Meunier, 2002; Hutchinson & Trueman, 2006) and makes up ~30% of the scale mass in some species (Neave, 1936). In summary, when considering the scale in planar view, the osseous layer contains material which decreases in age from the centre to the outer edge of the scale in a chronological fashion (Fig. 4). In contrast, the fibrillary plate consists of chronological material arranged vertically, decreasing in age with distance from the osseous layer (Fig. 4) (Hutchinson & Trueman, 2006). Growth of the scale is thought to start with the mineralisation of the osseous layer, followed by the growth of the basal plate (Fouda, 1979). Scale hydroxyapatite forms sequential ridges called circuli

(Fig. 3 and 4). No correlation has been found between the number of circuli and fish age, i.e. the circuli are not formed in any temporal pattern, based on findings in a study conducted by Fouda (1979) on the common goby (*Pomatoschistus microps*). However the width between sequential circuli can vary, producing widely and narrowly spaced circuli corresponding to an increase and decrease in fish growth respectively (Elliott & Chambers, 1996). As fish growth varies seasonally, alternating clusters of widely spaced and narrowly spaced circuli (corresponding to summer and winter growth respectively) can be used to determine the age of the fish in some species (Elliott & Chambers, 1996).

There are some disadvantages to using scales as biogeochemical tags. If scales are accidentally lost/scraped from the body of the fish, they are replaced by rapidly regenerated scales. These 'regenerated' scales do not contain the chronological hydroxyapatite formed prior to the removal of the original scale, i.e. in regenerated scales the lifelong chronological deposition of scale material is incomplete. The chronological deposition of hydroxyapatite in scales can also be partially resorped during periods of low Ca availability (Campana & Neilson, 1985). In anadromous species this typically occurs during migration to spawning grounds in freshwater as fish move from calcium-rich marine water to calcium-poor freshwater (Persson *et al.*, 1998). Regenerated and/or resorped scales can be easily identified under a microscope and avoided if necessary. However, it is possible that the hydroxyapatite component of scales might undergo continued crystallisation (Fouda, 1979) or even post-depositional change in composition once formed, which can disrupt the chronological record of element impurities (Fouda, 1979; Wells *et al.*, 2003b). While the degree to which post-deposition change occurs in scale hydroxyapatite is uncertain, scale hydroxyapatite has proven sufficiently stable to provide a non-lethally collectable biogeochemical tag for fish (Ophel & Judd, 1968; Yamada & Mulligan, 1982; Coutant & Chen, 1993; Adey, 2007; Adey *et al.*, 2009). Another advantage of scales is that their removal is a rapid, low-skill process which can be repeated throughout the lifetime of a fish (Adey *et al.*, 2009). Also, in contrast to otoliths, the chronologically deposited hydroxyapatite is exposed on the surface of the scale and so scales do not require the time-consuming sectioning and polishing that is needed to expose the growth bands in otoliths (Gillanders, 2001).

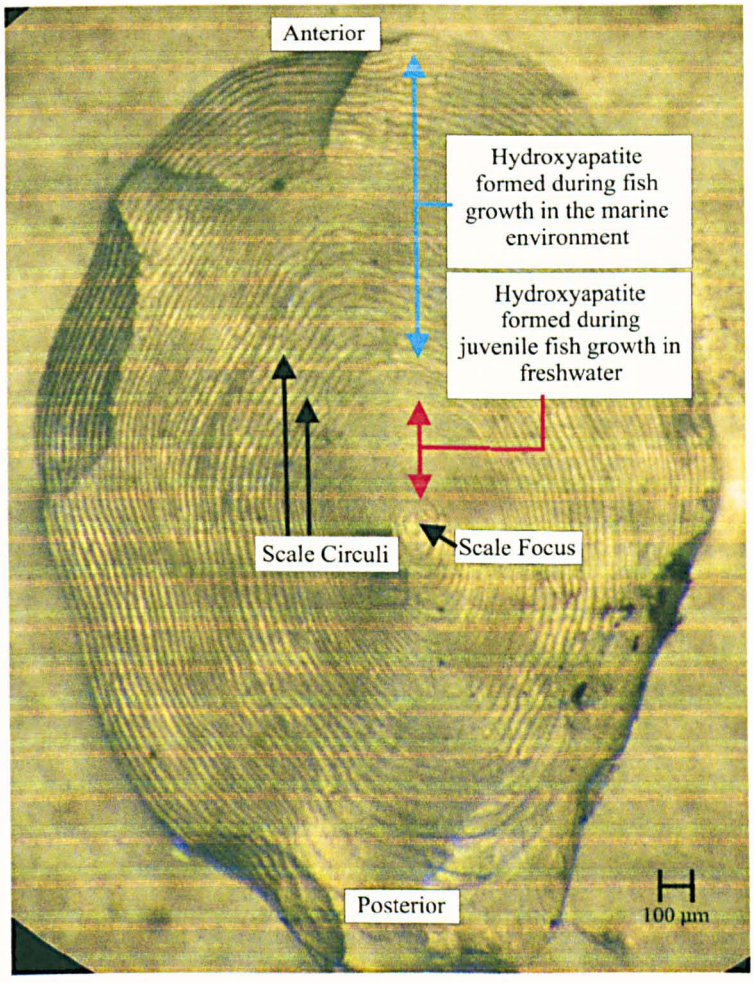

Figure 3. Image of a sea trout scale with the hydroxyapatite layer (External Layer; Fig. 4) facing upwards. The scale is annotated with characteristics which were determined through scale reading. Anterior and posterior regions of the scale are labelled using a similar nomenclature to that described by Meunier (2002). The image was captured using a camera mounted on a binocular microscope.

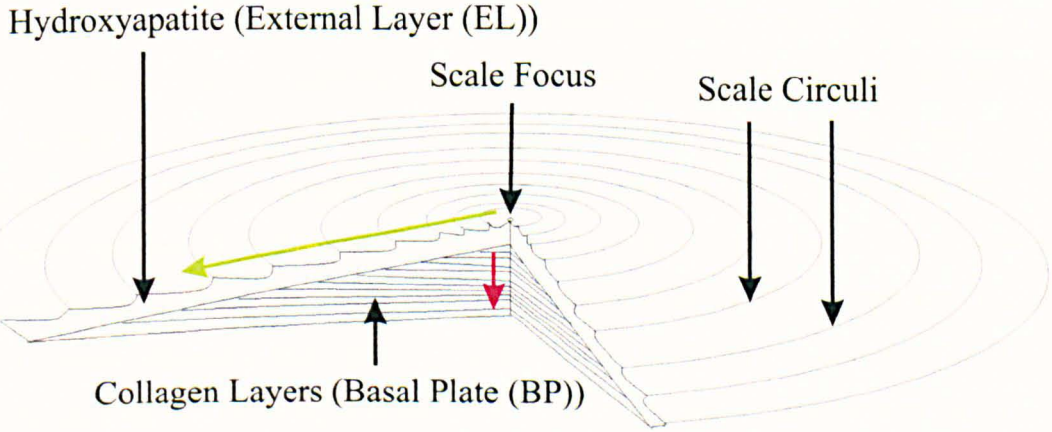

Figure 4. Hypothetical schematic of a *Salmo trutta* scale in cross-section, based on a diagram in Hutchinson & Trueman (2006). The chronological age of hydroxyapatite in the External Layer is indicated by the green arrow which points from 'old' to recently formed hydroxyapatite. The red arrow indicates the direction of the Basal Plate accretion and points from 'old' to recently deposited collagen.

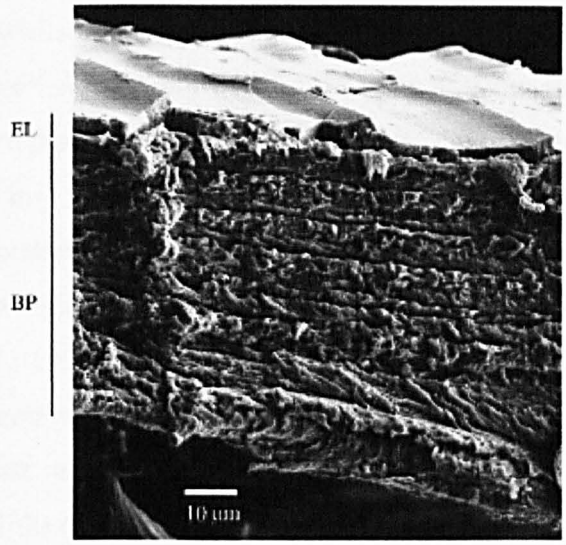

Figure 5. Scanning electron microscope micrograph of a vertical section through an Atlantic salmon (*Salmo salar*) scale [copied from Hutchinson & Trueman (2006)] which shows the collagen layers in the Basal Plate (BP) and the hydroxyapatite in the External Layer (EL).

Elements in water pass through several interfaces; uptake via the gills or the intestine, cellular transport and crystallisation, before being incorporated into otoliths (Elsdon & Gillanders, 2002) and scales. These interfaces may have the effect of regulating the uptake of elements in the otoliths and scales (e.g. by diluting or concentrating them). Scales receive elements directly from the bloodstream (Wells *et al.*, 2000b) whereas otoliths receive elements through a more regulated pathway, via the endolymph fluid in which the otolith is bathed (Campana, 1999). Otoliths and their surrounding endolymph fluid are contained in a semi-permeable inner ear membrane (Campana & Thorrold, 2001). Calcium carbonate crystallises out of the endolymph fluid onto the outer surface of the otolith. As the crystallisation of scale hydroxyapatite and otolith aragonite takes place, elements such as Sr, Mn and Ba are incorporated into the structures (Elsdon & Gillanders, 2002). Kalish (1989) found correlations between concentrations of Sr in the endolymph fluid and otoliths in 12 fish species (Kalish, 1989). Some elements, that have a similar valancy (2+) and ion radius to Ca (Ca^{2+}, 0.99 Å) such as Sr^{2+} (1.13 Å) (Radtke, 1989) and to a slightly lesser degree Ba^{2+} (1.36 Å), substitute for Ca in the matrices (Radtke, 1989; Campana, 1999; Gillanders, 2001) (forming e.g. $BaCO_3$ or $SrCO_3$ in otoliths) (Coillie & Rousseau, 1974; Elsdon & Gillanders, 2005). Because elements such as Sr and Ba substitute for Ca, their uptake

is proportional to the amount of Ca in water which explains why there is often a relationship between Sr:Ca or Ba:Ca in water and the ratios of these elements in fish calcified structures (Wells *et al.*, 2000a; Muhlfeld *et al.*, 2005). It is slightly less clear whether Mg (Mg^{2+} 0.65 Å) and Mn (Mn^{2+} 0.80 Å) substitute for Ca or whether they are trapped in interstitial spaces as these elements have a smaller ion radius compared to Ca (Hamer & Jenkins, 2007). There is evidence that some elements such as Cu and Zn bind to the proteinatious component of the structure (Miller *et al.*, 2006) whereas other elements are thought to become entrapped within the interstitial spaces in the matrix (Fowler *et al.*, 1995a; reviewed in Campana, 1999; Gillanders, 2001). Apart from the major elements which constitute aragonite (Ca, C and O) and hydroxyapatite (Ca, P, O, H), most elements occur in minor >100ppm and trace <100ppm concentrations in otoliths (Campana, 1999) and scales (Adey, 2007).

In a laboratory study examining the uptake of elements in calcified structures of fish, Wells *et al.* (2000a) found that the ratios of Sr:Ca and Br:Ca in otoliths and scales of the same fish [juvenile spot fish (*Leiostomus xanthurus*)] were highly correlated. Sr:Ca levels in otoliths and scales were similar, while Br:Ca and Mn:Ca levels were much higher in scales campared with otoliths. Similar results have been found elsewhere (Wells *et al.*, 2000b; Campana & Thorrold, 2001; Muhlfeld *et al.*, 2005). Elements may be present at lower concentrations in otoliths compared with other calcified structures (Clarke *et al.*, 2007) and presumably some elements which occur at levels below current analytical limits of detection are still awaiting detection (Campana, 1999).

A limited number of studies have compared the performance of biological structures in fish as biogeochemical tags (Wells *et al.*, 2003a; Courtmanche *et al.*, 2006; Clarke *et al.* 2007) and have generally concluded that otoliths offer a superior tag compared with other structures (Clarke *et al.*, 2007; Wells *et al.*, 2003a). However, past research has often focused on a small suite of element impurities that have a similar valency and ion radius to Ca and which might substitute for Ca in otolith aragonite (Campana, 1999) and scale hydroxyapatite (e.g. Sr, Ba and possibly Mn and Mg) (e.g. Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007). Recent evidence suggests that elements such as Li and U in scales might assist in discriminating the freshwater natal origins of wild fish (Adey, 2007). The crystal lattice of hydroxyapatite is less restrictive than that of aragonite and so the range of element impurities present in scales might be greater than in otoliths (Adey *et al.*,

2009). The range of elements available at the site of structure formation might also be greater in scales compared with otoliths, as scales receive elements directly from the bloodstream (Wells *et al.*, 2000b) whereas otoliths receive elements through a more regulated pathway via the endolymph fluid in which the otolith is bathed (Campana, 1999). Some elements are thought to become trapped in the interstitial spaces in the calcified matrix or bound to proteins in the organic component [e.g. Zn and Cu (Miller *et al.*, 2006)](Campana, 1999). Irrespective of whether these elements in fish-structures correlate with ambient concentrations in the water, they might provide a viable tag if their concentrations differ in fish among geographical locations. Advances in the sensitivity of analytical techniques have allowed reliable measurements to be made of a wide range of trace elements in biological structures, thus broadening the suite of elements available for biogeochemical tagging studies. However, some elements in otoliths are at such low concentrations that they fall below the analytical detection limits (Campana & Thorrold, 2001). This might limit the discriminatory power of otoliths compared with scales. As otoliths are contained in a semi-permeable membrane which 'isolates' them physically and chemically from the local environment, they seem less ideally suited to reflecting changes in environmental conditions (Campana & Thorrold, 2001).

While it is not essential to understand the processes by which elements are incorporated into fish structures in order to use them as biogeochemical tags (Thorrold *et al.*, 1998b; Elsdon & Gillanders, 2003b), it is important to be aware of the physical, chemical and biological factors which can have a controlling effect on the availability and incorporation of elements into calcified structures in fish (Fowler *et al.*, 1995a; Fowler *et al.*, 1995b; Elsdon & Gillanders, 2002) as this is likely to aid the interpretation of fish structure chemistry. An overview of the factors affecting the incorporation of elements in fish calcified structures is provided in Appendix B.

Temporal stability

Ideally biogeochemical tags should be temporally stable. A number of studies have tested temporal stability of biogeochemical tags over both short timescales (among months within years) and long timescales (among years). In freshwater and estuarine environments, there is the potential for significant changes in river flows over a range

of temporal scales (hourly, daily, monthly, yearly, decadal etc.) and this can affect the concentrations of elements in water. Element concentrations in fish structures have shown temporal variability (Campana *et al.*, 2000; Hamer & Jenkins, 2007; Clark *et al.*, 2009). A number of studies have demonstrated inter-annual differences in element concentrations in fish structures collected from the same geographical location in estuaries in different years (Hamer & Jenkins 2007; Clark *et al.*, 2009). The longest temporal stability study examined stability in cod (*Gadus morhua*) otoliths which were re-sampled over periods of up to 13 years (Campana *et al.*, 2000). While significant differences in element concentrations were detected among years, the relative difference in element concentrations between locations among years was maintained for some elements (Campana *et al.*, 2000). Temporal studies on other species/habitats undertaken over shorter timescales (~2yrs) have also found inter-annual variation. While this has not necessarily been sufficient to completely confound differences in element concentrations among locations, the degree to which fish collected over several years can be distinguished among stocks is sometimes compromised (e.g. Gillanders & Kingsford, 2000). For instance, Gillanders and Kingsford (2000) found significant differences in elemental compositions (Sr, Ba and Mn) in otoliths of juvenile trumpeter (*Pelates sexlineatus*) among and within estuaries on the East coast of Australia between years. Fish were classified to their site of origin with a higher degree of accuracy when considering only one years worth of data when compared with when the data for both years were combined (Gillanders & Kingsford, 2000). Seasonal variability in element concentrations in water (Elsdon & Gillanders, 2006b) and fish otoliths (Sr and Ba) have also been documented (Swearer *at al.*, 2003; Walther & Thorrold, 2009).

Instability in element concentrations in fish structures has also been demonstrated in fish inhabiting freshwater environments (Thorrold *et al.*, 1998a; Muhlfeld *et al.*, 2005; Walther & Thorrold, 2009). However, Feyrer *et al.* (2007) found that despite significant differences in concentrations of Sr and Ba in otoliths of splittails (*Pogonichthys macrolepidotus*) collected from rivers among different years, the relative difference in the concentrations of these elements among rivers was fairly stable (Feyrer *et al.*, 2007). In terms of stable isotope ratios, ^{87}Sr:^{86}Sr in otoliths have been found to be stable among years (Kennedy *et al.*, 2000) although this is not always the case (Walther & Thorrold, 2009). $\delta^{18}O$ has also been found to vary inter-annually (Walther & Thorrold, 2009). Variability in element concentrations and stable isotope

ratios among years does not necessarily compromise the discriminatory power of biogeochemical tags in fish, providing that the origins of particular cohorts can be matched with the corresponding baselines for the appropriate year-class (Clark *et al.*, 2009; Walther & Thorrold, 2009).

Aims and Objectives of the Thesis

The aims and objectives of the current thesis are as follows:

1. To examine whether element concentrations in the hydroxyapatite of sea trout (*Salmo trutta*) scales, formed during juvenile growth in freshwater, can be used to determine the natal river catchment of origin of wild fish. Establishing a catchment specific element 'signature' in the scales of sea trout, might provide an opportunity to determine habitat use and movement patterns of the species in the relatively closed area of the Irish Sea and Celtic Sea.

2. To compare the degree to which scale hydroxyapatite and otolith aragonite record spatial variations in element concentrations in stream water chemistry and to examine whether scales might offer a non-lethal alternative to otoliths as biogeochemical tags of *Salmo trutta*. The degree to which fish can be classified to their site of origin, based on a broad range of element concentrations scales and otoliths, among sites in a small river catchment will be determined.

3. To evaluate the traditional site-based design for determining spatial variability in fish structure chemistry. This study will establish whether there is any regional patterning in *Salmo trutta* scale chemistry at study sites within a river catchment and whether this regional patterning is repeated at a fine spatial scale. Temporal stability in element concentrations will be examined by comparing variability in element concentrations in recently collected stream water samples and historical stream water samples.

4. To determine whether fish can be classified to their site of origin within a small river catchment, based on naturally occurring stable isotope ratios $\delta^{13}C$, $\delta^{15}N$ and δD in whole *Salmo trutta* scales. The accuracy of the classification will be compared with that achieved using element concentrations in scale hydroxyapatite of the same fish.

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Chapter 2

Can scale chemistry be used as a stock discrimination tool for sea trout (*Salmo trutta* L.) in the Irish Sea and Celtic Sea?

Abstract

Population declines of andromous *Salmo trutta* L. (sea trout) in the West of Ireland and in some areas of Scotland have highlighted the potential vulnerability of stocks in the species North Atlantic range. An understanding of the reasons behind the sea trout population declines has been hindered by a lack of information on the movement patterns, habitat use and inter-mixing of populations of the species, particularly in the marine environment. This study is investigating the feasibility of using element concentrations in the freshwater growth bands of the scales of sea trout as a tool for determining the natal river of origin of sea trout in the Irish Sea and Celtic Sea.

Statistically significant differences were found in the concentrations of Li, Mg, Mn, Sr, Ba and Pb in the hydroxyapatite freshwater growth bands of wild *Salmo trutta* scales collected from 12 catchments draining into the Irish Sea and Celtic Sea from the West coast of the U.K. While for some catchments fish could be classified to their catchment of natal origin with a high degree of accuracy (e.g. 92% of the Fyne sea trout), in other catchments the classification accuracy was much lower (e.g. 13% of the Nith sea trout) and when considering all of the catchments together only 50% of the fish studied could be correctly classified to their catchment of origin. Mis-classified fish were sometimes classified to the study catchments neighbouring the catchment of origin but there was little evidence of consistent regional patterning in scale chemistry. A comparison of element concentrations in scales from sea-run and freshwater-resident *Salmo trutta* suggested that there is some degree of post-depositional change in element concentrations in scale hydroxyapatite. The degree to which element concentrations in sea trout scales could be used as a stock discrimination tool of fish in the Irish Sea and Celtic Sea is probably limited.

Introduction

Fisheries research often involves the study of habitat use and stock composition of fish (Prager & Fabrizio, 1990; Campana *et al.*, 1999). Establishing the geographical locations inhabited by fish and the degree to which reproductively isolated populations inter-mix, provides key information for determining the vulnerability of fish species to declines in population numbers (Milton & Chenery, 2001; Walther & Thorrold, 2008).

Since the late 1980s, populations of the anadromous salmonid, sea trout (*Salmo trutta*), have suffered severe declines in the West of Ireland and since the early 1990s in some areas of Scotland (Dawson, 1998; Harris & Milner, 2006). A recent analysis of sea trout population monitoring data for the West of Ireland indicates that stocks have not yet recovered to pre-collapse levels (Gargan *et al.*, 2006). While sea trout stocks are likely to benefit from the recent closure of drift net fisheries in Irish waters and the phasing out of commercial fish netting elsewhere, the stock collapse in the 1980s and 1990s highlighted the vulnerability of sea trout in its native range (Harris & Milner, 2006). While the decline and lack of population recovery to date have been linked to infestations of sea lice (Gargan *et al.*, 2006), it has not been possible to assess accurately the threats to sea trout stocks because little is known about their habitat use, movement patterns and behaviour in the marine environment. This was highlighted as a knowledge deficit at an International Symposium on sea trout in 2004 (Harris & Milner, 2006). Further research is required on salmonid population dynamics in the marine environment in order to determine where and how mortality occurs (Potter & Crozier, 2000).

Unfortunately, methodological limitations have restricted our understanding of the habitat use and movement patterns of anadromous fish species such as sea trout during their life history stages in the marine environment. Using artificial tags on fish is labour-intensive and expensive and can be limited in its application due to relatively high mortality rates of juveniles at early life stages (Gillanders, 2005). The low recapture rates of tagged fish restrict the amount of data that artificial tagging can generate (Gillanders & Kingsford, 2000; Gillanders, 2005). An alternative approach is to use 'natural tags', such as morphometric (Haas-Castro *et al.*, 2006) or genetic differences (Bembo *et al.*, 1996) between populations of a species or differences in the element and isotope composition of fish-structures such as otoliths (Walther & Thorrold, 2008), scales (Adey *et al.*, 2009) and vertebrae (Mulligan *et al.*, 1983). The

chemical composition of fish-structures can reflect geographical variability in element and isotope compositions in water (Walther & Thorrold, 2008). Chemical analysis of discrete growth bands in these structures can provide chronological records of the chemically-distinct geographical locations inhabited by fish prior to capture.

The use of fish-structure chemistry as a fisheries research tool has generated much interest (reviewed in Campana, 1999; Wells *et al.*, 2000; Walther & Thorold, 2008). In some cases fish-structure chemistry has provided a superior stock discrimination tool when compared with conventional tagging techniques. Campana *et al.* (1999) found that although genetic analysis and artificial tagging of fish had failed to distinguish between stocks of cod (*Gadus morhua*) from different feeding grounds, element concentrations in the otoliths of cod provided a powerful stock discrimination tool. In a more recent study, element concentrations in otoliths of splittail (*Pogonichthys macrolepidotus*) in the San Francisco Bay Estuary classified fish to four natal catchments when only two populations could be distinguished using genetic techniques (Feyrer *et al.*, 2007).

In salmonid research, structure chemistry has proven successful in distinguishing between farmed and wild populations of the Atlantic salmon (*Salmo salar*) with a high degree of accuracy (Adey *et al.*, 2009). It has even been possible to distinguish between fish from particular farms (Adey *et al.*, 2009). Differences in structure chemistry have also been detected in wild Atlantic salmon (*Salmo salar*) populations originating from different natal catchments (Veinott & Porter, 2005; Adey, 2007). This chapter investigates whether the technique can be used to elucidate the natal catchment of wild sea trout. If successful, this could be used to reveal the habitat use, movement patterns and the inter-mixing of catchment-specific populations of sea trout in the marine environment. The technique has already shown promise as a tool for distinguishing between sea-run *Salmo trutta* and freshwater-resident *Salmo trutta*, based on the concentrations of Sr in scales (Bagenal *et al.*, 1973).

While the majority of studies on fish-structure chemistry have used otoliths (reviewed in Campana, 1999; Gillanders, 2005; Walther & Thorrold, 2008), research has shown that scales might offer a non-lethal alternative (Flem *et al.*, 2005; Adey *et al.*, 2009; Chapter 3). Both scales and otoliths grow incrementally with the sequential accretion of hydroxyapatite in scales and aragonite in otoliths (Campana & Neilson, 1985; Clarke *et al.*, 2007). However, unlike the aragonite structure of otoliths, there is evidence that scale hydroxyapatite might undergo continued crystallisation or even

post-depositional change (Fouda, 1979) which can disrupt the chronological record in the hydroxyapatite growth bands. The extent to which post-depositional change occurs is uncertain. In a pilot study for this thesis on the scale chemistry of three sea trout, significant differences were found in the concentration of Sr in scale hydroxyapatite deposited during freshwater and marine residency (One–way ANOVA $F_{1,13}$=20.25, P=0.001; $F_{1,10}$=56.61, P<0.001 and $F_{1,11}$=9.09, P=0.013) (Appendix C). This suggests that hydroxyapatite deposited during juvenile growth in freshwater is not completely 'overprinted' while the fish subsequently resides in the marine environment. This supports findings by Adey *et al.* (2009), who noted an abrupt change in the element composition of *Salmo salar* scales corresponding to the migration of fish from freshwater to the marine environment. Despite the possibility of post-depositional change, the chemical composition of scale hydroxyapatite has proven sufficiently stable to provide a useful tag of the geographical origins and movement patterns of some fish species (Ophel & Judd, 1968; Yamada & Mulligan, 1982; Coutant & Chen, 1993; Adey, 2007; Adey *et al.*, 2009).

The main aim of this chapter is to determine whether the element composition of sea trout scale hydroxyapatite, formed during juvenile growth in freshwater, can be used to determine the natal river catchment of wild fish caught in the Irish Sea and Celtic Sea. It is thought that sea trout tend to feed in coastal waters (Berg & Berg, 1987; Harris & Milner, 2006), thus the populations contributing to the mixed-stocks in the Irish Sea and Celtic Sea could be composed of sea trout from rivers discharging into the region. Establishing a catchment-specific element 'signature' in the scales of sea trout, might provide an opportunity to determine habitat use and movement patterns of the species in the relatively closed area of the Irish Sea and Celtic Sea. The bedrock geology on the West coast of the United Kingdom ranges from largely metamorphic bedrock in Scotland to Carboniferous and Triassic in England and Cambrian and Silurian in Wales. In Ireland the bedrock includes Carboniferous and Silurian geology. This degree of heterogeneity of bedrock geology could generate regional variability in element concentrations in stream water (BGS, 1999) and thus in sea trout scale chemistry during juvenile growth in freshwater. A second aim of this study will be to examine whether there is evidence of post-depositional change in *Salmo trutta* scale chemistry by comparing the element concentrations of the freshwater growth bands of sea trout scales from two catchments with scale chemistry in freshwater-resident individuals from the same catchments.

Method

Sea trout tend to return to their natal catchment to spawn with only a small proportion of individuals 'straying' to other rivers (Sambrook, 1983; Le Cren, 1985). Therefore, catching fish on return to their natal catchment provides an opportunity to examine fish of known natal origin. The majority of fish scale samples used in this study were collected from fish caught in river catchments in 2007. Scale samples were removed from sea trout from 12 major sea trout rivers discharging into the Irish Sea and Celtic Sea (Fig. 1, Table 1). Scales were either collected by anglers or were donated by the Environment Agency and Fisheries Rivers Trusts. Unfortunately it was not possible to collect sea trout scale samples from rivers on the East coast of Ireland in 2007, due to a ban on sea trout angling that year (W. Roche, Central Fisheries Board (CFB), Ireland, *pers. comm.*, 2007). However, scales from *Salmo trutta* smolts which had been collected in 1993 from four catchments on the East coast of Ireland were donated by the Central Fisheries Board, Ireland. Because of the difference in the timing at which these scale samples were collected and the different life-stage of the fish, scale element concentration data have been analysed separately and are presented in Appendix D.

The chemical composition of fish structures is known to vary among geographical locations within freshwater catchments (Muhlfeld *et al.*, 2005; Chapters 3 and 4). To account for potential intra-catchment variability in element concentrations in scales, the scale samples used in this study were removed while fish were in the main river channel of each catchment and should therefore represent the range of element concentrations in scale hydroxypatite formed during juvenile growth in the various tributaries of each catchment.

There is evidence that the chemical composition of fish-structures formed at a given location can vary among years (Wells *et al.*, 2000). In this study, scale samples from fish of a range of ages were collected in order to allow for potential inter-annual variability in scale chemistry within each catchment (Table 1). *Salmo trutta* are repeat spawners and have been found to survive as many as 11 (or more) spawning visits to their natal catchment (Harris & Milner, 2006). The collections made in 2007 represent fish which resided in freshwater in different years (Table 1). This should allow for

potential inter-annual variation in baseline element signatures within each catchment. The years in which the hydroxyapatite was formed in the sea trout scales collected in 2005 from the Douglas river (Table 1) overlap with the range of years during which the hydroxyapatite was formed in the sea trout scales collected from catchments in 2007 (Table 1).

Scales samples were removed from the left side of the fish, above the lateral line, slightly posterior to the dorsal fin and stored in paper envelopes. Some scales were probably removed from the fish using metal instruments. However, research by Adey (2007), suggests that handling the scales with metal instruments at this stage is unlikely to influence subsequent elemental analyses.

The element composition of the hydroxyapatite of the scales was determined using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). One undamaged, non-regenerated scale from each fish was prepared for analysis (Flem *et al.*, 2005). The samples were randomised to create blocks of scales, each of which contained one randomly chosen fish from each site. The scales were mounted onto glass slides using double sided tape (No More Nails Tape), so that the hydroxyapatite layer was exposed for LA-ICP-MS analysis. The tape had previously been analysed by LA-ICP-MS to confirm that elements of interest were either low or below detectable levels (Appendix A and E). Scales were manually cleaned under a dissecting microscope using nylon brushes and plastic-tipped forceps. These implements were acid-rinsed in 10% nitric acid (Trace Grade, Seastar Chemicals Inc.) between uses. Scale samples were then ultrasonically cleaned for 4min in trace element grade 3% hydrogen peroxide (Trace Grade, Fluka), triple-rinsed with 18.2MΩ water (Milli-Q) and dried overnight in a laminar flow hood. All equipment in contact with the scale samples was new for this experiment, acid-washed in 10% nitric acid (TraceMetal Grade, Seastar Chemicals Inc.), triple-rinsed in 18.2MΩ water, dried in a laminar low hood and stored in sealed plastic bags prior to use.

Element concentrations in the scale hydroxyapatite growth bands that were formed while the fish were in their first year of juvenile growth in freshwater, were analysed using LA-ICP-MS. The analysis was carried out at the NERC ICP-MS facility (Kingston University), using an Agilent 7500c Series ICP-MS Octopole Reaction System coupled to a Cetac LSX-100 (wavelength 266nm) laser. The energy of the laser was set so as to allow the removal of the hydroxyapatite layer of the scale from the collagen layer underneath. It is possible that some of the collagen layer might

also have been ablated (Wells *et al.*, 2000). The scan speed was set at 10μm/s and the firing repetition rate was set at 10Hz. The beam diameter was ~10μm. The analysis was conducted using a zig-zag raster and the distance between lines was set at 10μm. The total area of the raster was ~100μm^2. The ICP-MS was set to an integration time of 0.12s for each element. Blanks were measured before each ablation by running the ICP-MS for ~10s prior to initiating the laser. National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 610 was used to calibrate the facility. NIST SRM 612 was analysed approximately every 12 scale samples and NIST SRM 610 was analysed three times daily. The following isotopes were included in the analysis: ^{7}Li, ^{24}Mg, ^{44}Ca, ^{55}Mn, ^{59}Co ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{138}Ba, ^{208}Pb and ^{238}U.

The raw LA-ICP-MS data were processed offline using SILLS software (Mineralogical Association of Canada Short Course 40, Vancouver, B.C.). A plot of counts per second (CPS) for each isotope recorded during each ICP-MS run was used to isolate the CPS corresponding to scale hydroxyapatite removed during the ablations. Raw data were blank-corrected. The Limit of Detection (LOD) was calculated as the blank value plus three standard deviations (SDs) of the blank (Miller & Miller, 1993). Ca was used as an internal standard because it is a major component of the hydroxyapatite layer in scales and typically its concentration varies by less than 1% (Clarke *et al.*, 2007). Hydroxyapatite is not a stoichiometric crystal and so a value of 374,000 μg/g (±4,000μg/g) was used for the Ca content of scale hydroxyapatite (as reported by Flem *et al.*, 2005). Concentrations of the remaining elements in the analysis were expressed in relation to this and reported in μg/g of hydroxyapatite (Flem *et al.*, 2005).

Blank subtraction occasionally resulted in some negative values for a given element due to the CPS of the sample being lower than the blank. In these cases, a positive constant corresponding to the largest negative value +1 was added to all values for that element to allow $\log_{10}$ transformation (Fowler *et al.*, 1995). Outliers were identified as values greater than five standard deviations away from the mean for that element (Veinott & Porter, 2005) and were replaced with the mean value of that element, pooled across all sites. For the majority of elements <2% of the data were classed as outliers and these were distributed evenly among samples pooled across all sites. Just less than 4% of Sr values in scales were classed as outliers. However, as almost all of these Sr values were for scales from the Fyne and are probably due to

relatively high levels of Sr at this site, the original scale Sr data was therefore kept in the analysis.

Analytical precision during the scale analysis was calculated as the relative standard deviation (RSD) of element:Ca in the NIST SRMs. Precision for the NIST SRM 610 and 612 during analyses was as follows (shown as element followed by RSD (%) for NIST 610/612): Li 10/5, Mg 9/14, Mn 5/5, Co 9/6, Cu 32/42, Zn 13/11, Sr 4/3, Ba 4/4, Pb 10/6 and U 10/7. No systematic drift was detected during the analysis. Recovery of element concentrations in the NIST 610/612 during analysis was determined in relation to the published element concentrations in NIST 610 and NIST 612 (Pearce *et al.*, 1997) and were as follows (shown as percentage recovery of each element for NIST 610/612): Li 100/99, Mg 113/99, Mn 98/96, Co 96/95, Cu 71/91, Zn 78/96, Sr 100/101, Ba 104/101, Pb 101/99 and U 103/104.

The percentage of the scale data (pooled across sites), for which element concentrations fell below the LOD were as follows: Pb 10%, Li 48%, Cu, 49%, Co 77% and U 68%. Less than 2% of the data for the remaining elements were below the LOD. Co and U were removed from further statistical analysis because over 50% of the data fell below the LOD.

Log_{10} element concentrations were tested for normality using Anderson-Darling's (AD) test. All log_{10} element concentration data failed normality tests. However, graphical inspection of the element data revealed relatively minor deviations from normality (McGuinness, 2002). The log_{10} element concentration data were also tested for homoscedasity using Levene's test. Li, Cu, Zn, Ba and Pb data failed homoscedasity tests.

Element concentrations in calcified structures have been shown to vary with fish length/otolith size (Thorrold *et al.*, 1998; Brophy *et al.*, 2003). In this study, fish fork length was used to examine the effect of fish size/age on element concentrations. ANCOVA was used to reveal common within-group regression slopes of element concentration and fork length at each site by using each element in the scales as the response variable, site as a category variable and fork length as a covariate. Providing that the assumption of equal slopes across sites was met, the effect of fork length on element concentrations was removed by using the common within-group regression coefficient in the following equation, adapted here from Almeida *et al.* (2008):

$$AC_{ij} = UAC_{ij} - [\beta \times (Log_{10}FL_j - Log_{10}\underline{FL})];$$

where AC_{ij} is the adjusted transformed element concentration for element *i* of the *j* specimen, UAC_{ij} is the unadjusted character measurement for element *i* of the *j* specimen (UAC was $\log_{10}$ transformed), β is the equal common within-group regression coefficient of element *i* ($\log_{10}$ transformed) regressed against fork length ($\log_{10}$ transformed), FL_j is the fork length of the *j* specimen ($\log_{10}$ transformed) and $\underline{FL}$ is the mean fork length of all fish ($\log_{10}$ transformed).

The ANCOVA was repeated (this time with the adjusted element data as the response variable) to confirm that the regression slope between fork length and element concentration data was no longer significant ($P>0.05$). Within-site regressions of adjusted element concentrations were used to confirm that length was no longer a significant covariate. The adjusted element data were used in all subsequent analyses. ANCOVA is robust to departures from normality providing data are homoscedastic (Olejnik & Algina, 1984) and it has been used to correct the effects of size on non-normal data (Claytor *et al.*, 1991; Almeida *et al.*, 2008). The Log_{10} element concentration data for some elements were both non-normal and heteroscedastic, however, ANCOVA has been demonstrated to still be robust provided that sample sizes are large (Olejnik & Algina, 1984).

Log_{10} element concentrations in the freshwater growth bands of the scales were examined for significant differences among catchments. One-way ANOVA and Scheffe's post hoc tests were used when $\log_{10}$ element concentration data were homoscedastic and Kruskal-Wallis and Tamhane's post hoc tests was used were data were heteroscedastic.

The accuracy with which fish could be classified to their catchment of origin based on element concentrations in scales, was determined using Linear Discriminant Function Analysis (LDFA). The LDFA involved an 'original' classification (which used the discriminant functions to classify the same samples that were used to develop the functions) and a 'cross-validation' (CV) classification (which involved leaving one sample out of the dataset before establishing the discriminant functions and then classifying the sample that was removed). As some of the $\log_{10}$ element concentrations violated assumptions for parametric tests, a cautionary approach was adopted and non-parametric Multinomial Logistic Regression (MLR), was used to validate the overall and group classifications achieved with the LDFA and to monitor the potential effects of non-normality on the LDFA results. Collinearity was detected among elements and

therefore a stepwise LDFA was avoided and non-stepwise LDFA Standardised Discriminant Function Coefficients used to indicate the relative importance of element concentrations in the LDFA classification (Sharma, 1996; Milton & Chenery, 2001).

Canonical Variate Plots provide a visual representation of the classification of individual fish to their site of origin. Cohen's Kappa statistic was used to compute the chance-corrected agreement between actual and predicted group (catchment) memberships of fish (Titus *et al.*, 1984; Barnett-Johnson *et al.*, 2008). The Kappa statistic ranges between 0 (indicating the classification to catchment was no improvement over that achieved by chance) and 1 (indicating that there was perfect agreement in the classification to catchment when taking into account classification by chance).

In order to examine whether post-depositional change occurs in hydroxyapatite, element concentrations in the freshwater hydroxyapatite growth bands of adult sea trout from the Nith catchment in Scotland were compared with element concentrations in the freshwater growth bands of smolts (i.e. fish which have undergone juvenile growth in freshwater but have not yet migrated to the marine environment) from the same catchment. Similarly, data on the element concentrations in scales of freshwater-resident *Salmo trutta* from the Dee catchment were compared with scales from adult sea trout from the Dee catchment. The scales of freshwater-resident *Salmo trutta* were analysed as part of a separate study which was carried out on a different LA-ICP-MS to the one used in this study. While values will probably differ due to inter-facility differences (Campana *et al.*, 1997), a comparison of sea trout scale samples which were analysed on *both* LA-ICP-MS facilities, revealed a close agreement between them (Table 2). Details of the scale sample preparation and analytical procedures for scales of freshwater-resident *Salmo trutta* from the Dee are reported in Chapter 3.

All statistical analysis was carried out in SPSS (v. 16) and MINITAB (v. 14).

Results

Sea trout scale samples were collected from 12 catchments on the West coasts of Scotland, England and Wales and from the Isle of Man (Fig. 1). Sample sizes of fish for each catchment ranged between 11 and 34 individuals (Table 1). The methods used

to capture the sea trout and the details of the fish fork lengths and fish ages are shown in Table 1.

Significant differences in fish fork length were found among the 12 catchments (Kruskal-Wallis; H=57.60, df=11, $P<0.001$). ANCOVA revealed a significant effect of fish fork length on $\log_{10}$Pb in scales (common within-group regression slope: $\beta=0.48$; $P=0.002$). The effect of fork length on Pb concentrations was removed and the adjusted data were used in the remaining statistical analyses.

Mean element concentrations in the freshwater growth bands of *Salmo trutta* scales from each of the 12 catchments are shown in Table 3. One-way ANOVA and Kruskal-Wallis revealed significant differences in the concentrations of all elements in sea trout scales among catchments, except for Cu and Zn. However, there was a significant difference in Zn concentrations between one pair of sites in the post hoc test (Table 3). Sr, Ba and Li were the elements that varied the most in concentration among sites (Scheffe's post hoc tests; Table 3) with 22, 29 and 17 pairs of catchments (out of a total of 66 pairs) showing statistically significant differences in the concentrations of Sr, Ba and Li respectively (Table 3).

50% of sea trout could be classified to their catchment of origin, using CV LDFA based on all elements which had shown a significant difference in concentration in scales among catchments using ANOVA or Kruskal-Wallis (i.e. Mg, Mn, Li, Sr, Ba and Pb) (Table 3). The Cohen's Kappa statistic was 0.44 (±0.6 CIs). The accuracy with which sea trout could be correctly classified to specific natal catchments ranged between 13% (Nith) and 92% (Fyne) (Table 4). Sr was the most important element in discriminating the catchment of origin of sea trout, as indicated by the Standardised Canonical Discrimnant Function Coefficients in which Sr had the highest absolute value in the first function, which explained a high proportion of the variance (Table 5). The overall MLR and LDFA ('original') classification accuracies were the same, indicating the non-normally distributed and heteroscedastic element concentration data did not effect on the LDFA results in this study (Table 6). The canonical variate plot (Fig. 2) shows that while some catchments appear to have quite distinct element 'fingerprints' in the scales of sea trout (e.g. Fyne and the Taff), many of the catchments cannot be clearly differentiated based on the first two discriminant functions [which explained 84% of the total variability in scale chemistry among catchment (Table 5)].

Mis-classified fish were not generally classified to the nearest study catchment neighbouring their true catchment of origin and this indicates that there is little regional patterning in the element concentrations in the freshwater growth bands of sea trout in this study (Table 4). However, when the CV LDFA classification accuracy was adjusted to include fish which were classified to their natal catchments *or* to the study catchments neighbouring their catchment of origin, the classification did improve to 66% (Table 4).

For some elements, significant differences were found in the concentrations in the freshwater growth bands of adult sea trout scales from the Nith and Dee catchments when compared with freshwater-resident fish from the same catchments (Table 7). For instance, Sr concentrations were generally higher in scales of sea trout compared with fish which had only resided in freshwater from both the Dee and Nith catchments (Table 7). The range of Sr values in freshwater and marine dwelling *Salmo trutta* did not overlap in the Dee fish which suggests that some degree of post-depositional change might have occurred. However, there was overlap in the ranges of Sr in the Nith fish and in both the Dee and Nith fish for some of the other elements (e.g. Ba).

Discussion

The results of this chapter have shown that concentrations of Mg, Mn, Li, Sr, Ba and Pb in the hydroxyapatite freshwater growth bands of wild sea trout scales varied among fish from 12 catchments draining into the Irish Sea and Celtic Sea. While elements such as Mg, Mn, Sr and Ba are typically used in biogeochemical tagging studies (Muhlfeld *et al.*, 2005; Veinott & Porter, 2005), this study shows that Li and Pb might also provide useful biogeochemical tags. However, while some fish were classified to their catchment with a high degree of accuracy (e.g. 92% of Fyne sea trout), overall, based on element concentrations in scales, only 50% of all fish included in the study could be correctly classified to their natal catchment of origin. The Cohen's Kappa statistic was relatively low 0.44 (±0.6 CIs), indicating that there is only moderate agreement between the actual and predicted classification of fish to catchment of origin when taking into account classification which could have occurred by chance.

From a fisheries management perspective, the level of acceptable error in classifying fish origin is probably dependent to a certain extent on the nature of the research question to be addressed. It is difficult to compare the relative success of biogeochemical tags as a fisheries research tool because the majority of studies do not establish baseline 'signatures' of all sources of fish contributing to the mixed stock (e.g. Wells *et al.*, 2000; Muhlfeld *et al.*, 2005; Veinott & Porter, 2005). In these instances, the accuracy with which fish can be classified to their stock of origin should be considered in relation to the proportion of the total number of sources for which baseline element signatures have been determined (Gillanders, 2005), but this is seldom reported. It was beyond the scope of the current study to sample fish-structure chemistry at all geographical locations contributing sources of fish to the mixed–stocks in the Irish Sea and Celtic Sea. This would have required sampling the scale chemistry of sea trout in >80 major sea trout catchments discharging into the region and would have assumed that these catchments alone were responsible for contributing fish to the mixed-stock in the Irish Sea and Celtic Sea. Given that in this study 12 catchments (of a possible >80 catchments) contributing fish to sea trout stocks in the Irish and Celtic Sea were included in the analysis, the classification accuracy of only 50% (of which a significant proportion would have been due to correct assignment of fish to catchment of origin by chance alone), indicates that the technique has limited application as a fisheries research tool for determining catchment of origin of *Salmo trutta* in this geographical region.

This study probably provides a relatively conservative estimate of the degree to which element concentrations in the hydroxyapatite growth bands of scales of *Salmo trutta* vary among catchments, for several reasons. Although the degree to which any post-depositional change might have occurred on re-entry of the fish to freshwater is unclear, the majority of post-depositional change in the freshwater element 'signature' in scales might already have taken place while the sea trout were residing in the marine environment. The scale chemistry of the fish in this study represent the potential differences in the element concentrations in scales at different spawning sites within each catchment (Mulhfeld *et al.*, 2005) given that the sea trout were caught in the main river channels of each catchment. Inter-annual changes in the baseline chemical signatures within each catchment were accounted for by collecting fish of a range of ages. Finally, an estimate of the influence of classification of fish to their catchment of origin by chance, was provided.

The relatively poor accuracy with which fish could be classified to their catchment of origin might be due to a combination of factors. It has been shown that significant differences in fish-structure chemistry can occur among sites within a catchment (Muhlfeld *et al.*, 2005; Chapters 3 and 4). If intra-catchment variability in fish structure chemistry is high relative to inter-catchment variability, this would compromise the accuracy with which fish could be classified to their catchment of origin. The degree of intra-catchment variability in *Salmo trutta* scale chemistry will be explored in two further studies (Chapters 3 and 4).

An alternative reason for the poor accuracy could be post-depositional change in the element composition of hydroxyapatite in scales (Fouda, 1979; Wells *et al.*, 2003). In this study, the range of element concentrations in the freshwater growth bands of sea trout scales returning to freshwater after residing in the marine environment differed significantly compared with that of the scales of freshwater-resident fish, suggesting that post-depositional change in hydroxyapatite had taken place. Providing that post-depositional change occurs in a relatively uniform fashion and does not completely overprint the freshwater 'signature', the relative difference in the chemistry of the freshwater growth bands of scales from each catchment should be preserved (even if absolute concentrations are not). Given that 1) the pilot study found significant differences in the element concentrations of hydroxyapatite deposited during marine and freshwater residency in the same scale and 2) this study found significant differences in the element concentrations in the freshwater growth bands of sea trout scales, it seems that the freshwater 'signature' is not completely overprinted. This supports existing evidence in the literature that a freshwater signature in scale hydroxyapatite is preserved even after fish have been residing in the marine environment (Adey *et al.*, 2009).

It is possible that the sea trout scales collected for this study originated from fish which had strayed from their natal catchments. This could have interfered with the element 'signature' determined for each catchment. While it was not possible to quantify the number of 'stray' sea trout in this study, evidence from tagging studies indicate that a relatively small proportion of sea trout enter non-natal catchments during the spawning run (Sambrook, 1983; Le Cren, 1985), suggesting that stray fish are unlikely to have heavily influenced the results of this study.

This study suggests that there is little regional patterning in scale chemistry in U.K. catchments and therefore the extent to which adult *Salmo trutta* scale chemistry

could be used as a biogeochemical tag of Irish Sea and Celtic Sea sea trout is probably very limited. However, given that there are significant differences in a number of element concentrations in scales from different catchments, the technique might contribute to the discrimination of fish populations if it were combined with other stock discrimination techniques, such as genetic (Bembo *et al.*, 1996) or morphometric stock discrimination tools (reviewed in Begg & Waldman, 1999).

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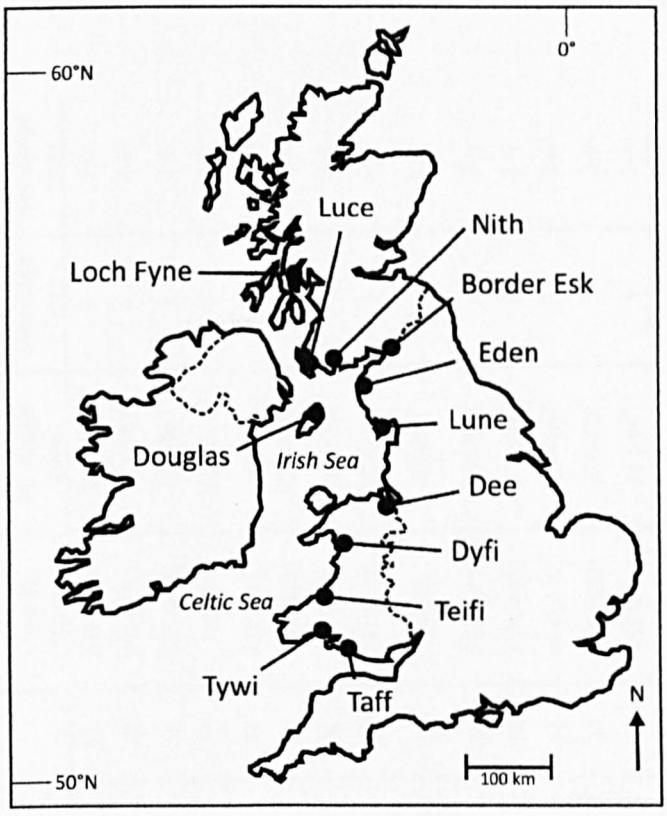

Figure 1. Map of the U.K. showing the river catchments from which sea trout (*Salmo trutta*) scale samples were collected. Information on the fish samples from each catchment can be found in Table 1.

Table 1. Site locations, sample sizes (n) and details of the fork lengths of *Salmo trutta* collected from 12 catchments on the West coast of the U.K. Scale samples were collected from *Salmo trutta* in 2007, except for fish from the Douglas river which were collected in 2005. Fish age refers to the combined freshwater and sea age, i.e. the total age of the fish. The average duration of juvenile freshwater residency in each catchment was 2 years. *Denotes scale samples analysed on a separate LA-ICP-MS (see Methods section). **Netting was carried out in Loch Fyne near the mouth of the Fyne river.

Catchment	Country/Region	Maturity Status	Capture Method	n	Mean FL (±SE) (mm)	FL Range (mm)	Mean Age (yrs)	Age Range (yrs)
Fyne	Scotland	Sea trout	Netting**	12	309 (±20)	200-405	4	3-6
Luce	Scotland	Sea trout	Angling	12	501 (±30)	381-787	4	3-9
Nith	Scotland	Sea trout	Angling	16	413 (±18)	440-460	3	2-4
		Smolt	*Electrofishing*	*12*	*140 (3)*	*125-170*	*2*	*2-2*
Border Esk	Scotland/England	Sea trout	Angling	34	422 (±11)	305-610	3	2-5
Eden	England	Sea trout	Fish Trap	12	480 (±28)	360-670	3	3-5
Lune	England	Sea trout	Fish Trap	30	503 (±12)	400-600	4	3-6
Dee	England/Wales	Sea trout	Fish Trap	27	391 (±23)	222-652	3	2-6
		*Freshwater-resident trout**	*Electrofishing*	*83*	*158 (4)*	*83-258*	*N.A.*	*N.A.*
Dyfi	Wales	Sea trout	Angling	24	507 (±17)	381-800	4	2-6
Teifi	Wales	Sea trout	Angling	14	470 (±35)	279-686	3	2-6
Tywi	Wales	Sea trout	Angling	11	414 (±46)	200-660	3	2-6
Taff	Wales	Sea trout	Angling	34	435 (±20)	320-770	5	2-8
Douglas	Isle of Man	Sea trout	Fish Kill	12	455 (±33)	287-647	3	2-6

Table 2. Comparison of the element concentrations in the freshwater hydroxyapatite growth bands of *Salmo trutta* scales from the Luce (sea trout A) and Border Esk (sea trout B) detected by two separate facilities (LA-ICP-MS A and B) to determine the similarity between scale element concentrations analysed on two separate facilities.

	Element concentrations in the freshwater growth bands of Sea trout scales (µg/g)			
	Sea Trout A		Sea Trout B	
Element	LA-ICP-MS (A)	LA-ICP-MS (B)	LA-ICP-MS (A)	LA-ICP-MS (B)
Mg	4313	4720	9563	11392
Mn	120	156	199	327
Co	0.38	0.37	0.28	0.67
Cu	2.51	3.98	1.56	2.56
Zn	862	906	530	457
Sr	1097	1150	1127	1465
Ba	8	10	20	27
Pb	203	215	1.4	2
U	0.15	0.16	0.04	0.05
Li	3.28	4.31	8.44	12.47

Table 3. Mean (µg/g±1SE) element concentrations in the freshwater growth bands of scales of sea trout from 12 catchments on the West coast of the U.K. (concentrations of Pb presented here are unadjusted for the effect of fork length on Pb concentrations; see Results). One-way ANOVA and Kruskal-Wallis test results on element concentrations in the sea trout scales are shown. *Denotes post hoc test carried out with Scheffe's test rather than Tamhane's test.

Element	Mean element concentrations in the freshwater growth bands of sea trout (*Salmo trutta* L.) scales from 12 catchments on the West coast of the U.K. (µg/g) (±1SE)												Anova (*F*) and Kruskal-Wallis (*H*)		Number of significant Post hoc pairs (*P*<0.05)
	Fyne	Luce	Nith	Border Esk	Eden	Lune	Dee	Dyfi	Teifi	Tywi	Taff	Douglas	Test statistic (df=11, 237)	*P*	
Li	5.8 (±1.2)	4.8 (±0.9)	1.4 (±0.9)	2.7 (±0.5)	9.3 (±1.4)	9.1 (±0.8)	4.5 (±0.9)	3.9 (±0.6)	2.0 (±0.6)	1.6 (±0.5)	0.9 (±0.2)	3.4 (±3.7)	*H*=86.88	<0.001	17
Mg	10252 (±635)	8783 (±398)	8812 (±923)	7545 (±444)	8066 (±806)	7297 (±492)	6977 (±602)	8748 (±671)	7683 (±466)	9100 (±940)	7492 (±479)	6187 (±635)	*F*= 2.38	0.008	0*
Mn	269 (±40)	265 (±36)	147 (±16)	134 (±13)	171 (±27)	133 (±14)	201 (±20)	179 (±19)	195 (±29)	195 (±38)	299 (±33)	204 (±35)	*F*=5.84	<0.001	2*
Cu	2 (±1)	2 (±0.3)	21 (±12)	5 (±1)	2 (±0.4)	2 (±0.5)	6 (±3)	3 (±1)	2 (±1)	1 (±1)	2 (±1)	55 (±26)	*H*= 14.86	0.189	0
Zn	416 (±30)	430 (±42)	603 (±114)	586 (±46)	600 (±111)	637 (±86)	524 (±45)	699 (±81)	579 (±77)	509 (±89)	720 (±64)	1122 (±304)	*H*=16.04	0.140	1
Sr	1780 (±62)	1229 (±29)	1048 (±53)	1075 (±35)	840 (±83)	1007 (±36)	1057 (±32)	1190 (±40)	950 (±30)	980 (±41)	584 (±34)	1114 (±56)	*F*=37.78	<0.001	22*
Ba	72 (±10)	31 (±4)	48 (±8)	48 (±4)	76 (±15)	52 (±11)	30 (±3)	19 (±2)	14 (±2)	24 (±2)	62 (±5)	18 (±3)	*H*=122.64	<0.001	29
Pb	3 (±0.5)	4 (±1)	8 (±3)	3 (±1)	9 (±3)	4 (±1)	6 (±1)	21 (±7)	5 (±1)	5 (±2)	7 (±1)	22 (±6)	*H*=34.07	<0.001	0

Table 4. Cross validation LDFA classification of sea trout to their catchment of natal origin based on concentrations of Li, Mg, Mn, Sr, Ba and Pb in the freshwater growth bands of scales. Cu and Zn were excluded from the LDFA because no significant differences were found in concentrations of these elements in scales among catchments using ANOVA (Table 3). Correctly classified individuals are shown in dark grey boxes and highlighted in bold. The light grey boxes correspond to study catchments neighbouring the natal catchments of origin. *Denotes the percentage of individuals correctly classified to their catchment of origin. #Denotes the percentage of individuals correctly classified to their natal catchment or study catchment neighbouring the natal catchment of origin.

	No. of sea trout classified to their catchment of natal origin based on element concentrations in the freshwater growth bands of scales														
Catchment	Fyne	Luce	Nith	B.Esk	Eden	Lune	Dee	Dyfi	Teifi	Tywi	Taff	Douglas	n	% Classified to natal catchment*	% Classified to natal catchment or neighbouring catchment of origin#
Fyne	**11**	1	0	0	0	0	0	0	0	0	0	0	12	92	100
Luce	0	**8**	0	1	0	1	0	0	0	2	0	0	12	67	67
Nith	0	0	**2**	**5**	1	0	3	1	0	1	2	1	16	13	44
B.Esk	0	1	**5**	**18**	**1**	6	1	0	0	0	1	1	34	53	71
Eden	0	0	1	**1**	**5**	**3**	1	0	0	0	1	0	12	42	75
Lune	2	2	0	1	**5**	**14**	**6**	0	0	0	0	0	30	47	83
Dee	0	6	2	1	1	**3**	**9**	**1**	1	2	0	1	27	33	48
Dyfi	0	2	2	0	0	0	**0**	**9**	**4**	1	0	6	24	38	54
Teifi	0	0	0	0	0	0	0	**2**	**9**	**2**	0	1	14	64	93
Tywi	0	0	2	1	0	0	1	2	**3**	**2**	**0**	0	11	18	45
Taff	0	1	1	0	1	0	2	0	0	**0**	**29**	**0**	34	85	85
Douglas	0	2	0	0	0	1	0	3	2	1	**1**	**2**	12	17	25

Table 5. Standardised Canonical Discriminant Function Coefficients for the LDFA performed on element concentrations in the freshwater growth bands of sea trout scales. The cumulative variance explained by the Functions, is also shown. Cu and Zn were excluded from the LDFA because no significant differences were found in concentrations of these in scales among catchments (Table 3). The most important element in the first LDFA function is underlined and shown in bold.

	LDFA Function				
Element	1	2	3	4	5
Li	0.06	0.24	-0.63	0.69	0.29
Mg	0.06	-0.30	0.20	0.01	0.29
Mn	-0.01	-0.62	0.58	0.70	-0.09
Sr	**0.91**	0.38	0.24	-0.05	-0.02
Ba	-0.32	1.08	0.31	-0.20	0.20
Pb	0.20	-0.44	0.02	-0.33	0.82
Cumulative variance explained (%)	47	84	91	95	98

Table 6. Original (i.e. non-Cross Validation) LDFA and Multinomial Logistic Regression (MLR) classifications of sea trout to their catchment of origin based on element concentrations (Li, Mg, Mn, Sr, Ba and Pb) in the freshwater growth bands of scales. Cu and Zn were excluded from the analysis because no significant differences were found in concentrations of these elements in scales among catchments (Table 3).

	Classification accuracy (%)	
Catchment	Original LDFA	MLR
Fyne	92	100
Luce	75	50
Nith	31	19
B.Esk	62	74
Eden	50	25
Lune	57	77
Dee	33	41
Dyfi	42	50
Teifi	86	64
Tywi	46	27
Taff	85	85
Douglas	50	33
Overall:	59	59

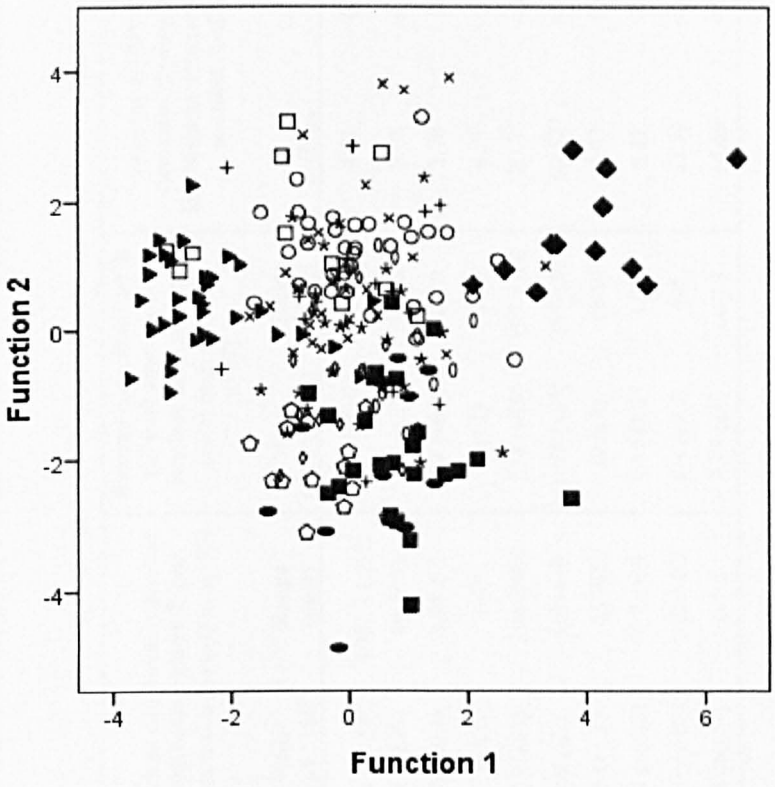

Figure 2. Canonical scores plot of the first 2 discriminant functions in the LDFA on Li, Mg, Mn, Sr, Ba and Pb in scales of sea trout from 12 catchments (Fyne, closed diamonds; Luce, stretched open squares; Nith (+); B. Esk, open circles; Eden, open squares; Lune (x); Dee, star; Dyfi, closed square; Teifi, open pentagon; Tywi, stretched open diamond; Taff, closed triangle; Douglas, stretched closed circle). Cu and Zn were excluded from the analysis because no significant differences were found in concentrations of Cu and Zn in scales among catchments (Table 3).

Table. 7. Evaluation of the stability of element concentrations in hydroxyapatite in *Salmo trutta* scales. Comparisons are made between the element concentrations in the freshwater growth bands of *Salmo trutta* which have spent a period of their life history in the marine environment (denoted sea trout) and *Salmo trutta* which have resided exclusively in freshwater (denoted freshwater-residents or smolts). *Salmo trutta* from the Nith catchment and Dee catchment are examined. Elements for which >50% of the data were <LOD are shown in italics. *Denotes Kruskal-Wallis test rather than ANOVA.

	Element concentrations in smolt scales from the Nith catchment (n=12)		Element concentrations in the freshwater growth bands of sea trout scales of the Nith catchment (n=17)		One-way ANOVAon element concentrations in Nith smolts (n=12) and sea trout (n=17)		Element concentrations in the freshwater-resident *Salmo trutta* scales in the Dee (n=83)		Element concentrations in the freshwater growth bands of adult sea trout scales from the Dee (n=27)		One-way ANOVA on element concentrations in Dee resident trout (n=83) and sea trout (n=27)	
Element	Mean (µg/g)(±1SE)	Range (µg/g)	Mean (µg/g)(±1SE)	Range (µg/g)	$F_{1,28}$	*P*	Mean (µg/g)(±1SE)	Range (µg/g)	Mean (µg/g)(±1SE)	Range (µg/g)	$F/H_{1,109}$	*P*
Mg	4822 (±524)	1760-7884	8495 (±922)	3020-15028	8.03	0.009	5229 (±270)	1731 - 10072	6978 (±602)	1266-12604	4.91	0.029
Mn	277 (±56)	105-832	199 (±54)	50-1026	0.98	0.332	211 (±19)	49-1031	202 (±21)	36-460	0.05	0.826
Co	*0.45 (±0.07)*	*0-0.8*	*0.7 (±0.20)*	*0.1-3.7*	0.71	0.408	1.2 (±0.1)	0.33-5.5	*0.8 (±0.4)*	*0-10*	2.76	0.1
Cu	6.3 (±2.4)	0-31	23 (±11.5)	0.4-189	1.48	0.235	7 (0.8)	1-49	6 (±3)	0-80	4.31	0.04
Zn	880 (±202)	372-2977	613 (±108)	43-2124	1.58	0.219	913 (±59)	296-3089	524 (±45)	197-1162	20.39	<0.001
Sr	556 (±27)	446-779	1066 (±52)	620-1352	69.12	<0.001	294 (±7)	167-449	1056 (±33)	755-1515	60.57*	<0.001
Ba	119 (±17)	67-237	52 (±8)	9-138	15.54	0.001	35 (±1.1)	15-82	30 (±3)	10-93	3.03	0.084
Pb	11 (±4)	1.3-43	15 (±7)	0-134	0.13	0.722	0.7 (±0.07)	0.7-965	4.5 (±1.2)	1-25	6.21	0.014
U	*0.45 (±0.23)*	*0-2.4*	*0.32 (±0.2)*	*0-3.09*	0.18	0.671	0.04 (±0.003)	0.005-0.2	*0.4 (±0.2)*	*0-6*	*52.36*	<0.001
Li	4 (0.6)	0.4-7	2.0 (±1)	0-11	1.67	0.208	1.4 (±0.13)	0-7.5	4.48 (±1)	0-13	14.69	<0.001

Chapter 3

Comparison of the performance of scale and otolith microchemistry as fisheries research tools in a small upland catchment

Abstract

LA-ICP-MS analysis of recently deposited *Salmo trutta* L. scale hydroxyapatite and otolith aragonite provided biogeochemical tags of *Salmo trutta* at 6 sites (>7.5km apart) in a small upland catchment (drainage area: ~1800km^2). 86% and 89% of fish were correctly classified to their site of origin based on Sr, Mn, Ba and Mg concentrations in scales and otoliths respectively. Sr, Mn and Ba were highly significantly correlated between structures of the same fish (P<0.001). Ba and Mn in both structures were significantly correlated with stream water chemistries at each site (P<0.05). Significant differences were found in 11 element concentrations in scales and 6 element concentrations in otoliths among sites (P<0.05). Broadening the suite of elements improved the classification of fish to site of origin to 88% for scales while there was no improvement in the classification for otoliths. When only Zn, Pb, Co, Cu, Si, Li and U in scales were used, the classification was 68%, suggesting that these elements could be important biogeochemical tags in future studies. There appears to be some degree of post-depositional change in scale hydroxyapatite. Scales might offer a non-lethal biogeochemical tag, comparable in performance to otoliths, but further work needs to examine the degree of post-depositional change in scale hydroxyapatite.

Introduction

Key techniques in fisheries research include tracking the movement patterns or provenance of fish. In recent years naturally occurring variations in element concentrations and stable isotope ratios in biological structures [e.g. vertebrae (Mulligan *et al.*, 1983), otoliths (Comyns *et al.*, 2008), scales (Wells *et al.*, 2003a), fin rays (Clarke *et al.*, 2007) and eye lenses (Gillanders, 2001)] have provided 'biogeochemical tags' for fish from different geographical locations (Campana, 1999). Although research has predominantly focused on the application of biogeochemical tags to the study of estuarine and marine fish (e.g. Swearer *et al.*, 2003; Gillanders, 2005; Comyns *et al.*, 2008), interest in the method for elucidating natal origins and movement patterns of freshwater species is increasing (Walther & Thorrold, 2008; Gibson-Reinemer *et al.*, 2009). Currently, the research indicates that the technique may have potential as a freshwater fisheries research tool in assigning the origins of wild freshwater fish to particular sites within large catchments (~5,000-100,000skm^2) (Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007; Adey, 2007). Another potentially interesting application of biogeochemical tags is in determining the migration patterns of fish in freshwater, however, the potential of the technique to resolve relatively small-scale movement patterns has yet to be determined.

While the native range of *Salmo trutta* is restricted to European waters, it has become established globally (Elliott, 1994) and might be a 'keystone' species in some freshwater ecosystems (Wilson & Halupka, 1995). *Salmo trutta* is an excellent model species for studies on biogeochemical tags, due to the continuum of life-history strategies that it exhibits. The species is phenotypically plastic and can inhabit freshwater for its entire lifespan, or utilise a combination of freshwater, estuarine and marine habitats (Jonsson, 1989; Cucherousset *et al.*, 2005). In recent years the migratory contingent of the species has suffered population declines in its native Scottish and Irish waters, the reasons for which are uncertain (Harris & Milner, 2006). Broadening our knowledge of the spatial ecology of the species could help to improve our understanding of potential threats to its populations. Conventional tagging data shows that freshwater-resident trout can move >20km during migrations to spawning grounds (Ovidio *et al.*, 1998; Rustadbakken *et al.*,

2004) but our knowledge of the extent to which individual fish exploit the river network accessible to them, in search of feeding and refuge habitats, is limited.

The otolith is the structure most commonly used in biogeochemical tagging studies, for several reasons. In contrast to other structures, its calcium carbonate structure starts forming prior to hatching and accretes metabolically inert, discrete seasonal or even daily growth bands throughout the lifetime of fish (Campana & Neilson, 1985). Element 'impurities' are incorporated into the growth bands as they form, creating a permanent chronological record of spatial variations of element impurities to which a fish has been exposed (Wells *et al.*, 2003a). Unfortunately, otolith collection necessitates the killing of fish which is particularly undesirable when studying rare species (Clarke *et al.*, 2007) and may reduce the market value of commercial catches (Gillanders, 2001).

Scales are composed in part, of hydroxyapatite, into which are incorporated element impurities during formation. The chronologically deposited hydroxyapatite is exposed on the surface of the scale, so does not require the time-consuming sectioning and polishing needed to expose the growth bands in otoliths and spines (Gillanders, 2001). Scale removal is a rapid, low-skill process which can be repeated throughout the lifetime of a fish (Adey *et al.*, 2009), allowing samples from a large number of fish to be collected and processed quickly. Unlike otoliths, however, scales can be partially resorped during periods of low Ca availability (Campana & Neilson, 1985) and might undergo continued crystallisation (Fouda, 1979) or even post-depositional changes in composition once formed, disrupting the chronological record of element impurities (Fouda, 1979; Wells *et al.*, 2003b). Nevertheless, scale hydroxyapatite has proven sufficiently stable to provide a biogeochemical tag for fish (Ophel & Judd, 1968; Yamada & Mulligan, 1982; Coutant & Chen, 1993; Adey, 2007; Adey *et al.*, 2009).

In a pilot study, we found that an abrupt change occurred in the element composition of sea-run *Salmo trutta* scales corresponding to fish movement between freshwater and marine habitats. Analysis of Sr in scales from three sea-run *Salmo trutta* showed that the scale hydroxyapatite growth bands formed while the fish were juveniles in freshwater, were significantly lower in concentration than those formed while the fish were subsequently residing in the marine environment (One–way ANOVA $F_{1,13}$=20.25, P=0.001; $F_{1,10}$=56.61, P<0.001 and $F_{1,11}$=9.09, P=0.013) (Appendix C). This indicates that a freshwater 'signature' is preserved in the

hydroxyapatite growth bands formed early in life, despite subsequent residence of fish in the marine environment. This finding supports previous work that indicated that Sr in *Salmo trutta* scales is stable (Bagenal *et al.*, 1973). Adey and co-authors have recently demonstrated that any post-depositional change that might have occurred in adult *Salmo salar* scales was insufficient to 'overprint' the element signature deposited during freshwater residency (Adey *et al.*, 2009).

A limited number of studies have already compared the performance of biological structures as biogeochemical tags in fish (Wells *et al.*, 2003a; Courtemanche *et al.*, 2006; Clarke *et al.*, 2007) and have generally concluded that otoliths offer a superior biogeochemical tag to scales (Wells *et al.*, 2003a; Clarke *et al.*, 2007). However, past research has often focused on a small suite of element impurities that have a similar valency and ion radius to Ca, e.g. Sr and Ba (Wells *et al.*, 2000b; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007) and which substitute for Ca in otolith aragonite (Campana, 1999; Martin *et al.*, 2004) and scale hydroxyapatite. However, recent evidence suggests that elements such as Li and U in scales might assist in discriminating the freshwater origins of wild fish (Adey, 2007). The crystal lattice of hydroxyapatite is less restrictive than that of aragonite and so the range of element impurities present in scales might be greater than in otoliths (Adey *et al.*, 2009). The range of elements available at the site of structure formation might also be greater in scales than in otoliths, as scales receive elements directly from the bloodstream (Wells *et al.*, 2000b) whereas otoliths receive elements through a more regulated pathway, via the endolymph fluid in which the otolith is bathed (Campana, 1999). Some elements are thought to be trapped in the interstitial spaces in the calcified matrix or bound to proteins in the organic component [e.g. Zn and Cu (Miller *et al.*, 2006)] (Campana, 1999). Irrespective of whether these elements in fish-structures correlate with ambient concentrations in the water, they might provide a viable tag of fish if their concentrations differ geographically. Advances in the sensitivity of analytical techniques have allowed reliable measurements to be made of a wide range of trace elements in biological structures, thus broadening the suite of elements available for biogeochemical tagging studies and possibly enhancing the discrimination power of scales, relative to otoliths.

The present study compares the performance of scales and otoliths as biogeochemical tags of *Salmo trutta* in a small catchment. The research was carried out in the Dee catchment in North Wales (drainage area: ~1800km^2), which is an

important *Salmo trutta* habitat in the U.K. Element concentrations in stream water in the region are known to vary considerably (BGS, 1999). This variation might generate differences in fish-structure chemistry over small spatial areas. Using a rigorous analytical approach, this study evaluates how well scales perform as tags of geographical origin relative to otoliths of the same fish by; i) comparing the degree to which scales and otoliths record spatial variability in element concentrations in relation to stream water chemistry; ii) comparing the extent to which scale and otolith chemistry varies between fish from different geographical locations (among sites >7.5km apart); iii) comparing the degree to which fish can be classified to their site of origin based on a broad range of element concentrations in scales and otoliths and; iv) examining if any evidence of post-depositional change in *Salmo trutta* scale chemistry exists when comparing the element concentrations of the freshwater growth bands of sea-run *Salmo trutta* with scale chemistry of smolts or freshwater-resident individuals from the same natal catchment.

Methods

Freshwater-resident *Salmo trutta* L. (n=83) were collected from 6 sites in the Dee catchment between August and September 2007. The sites were chosen to provide a geographical spread within the catchment (Fig. 1). Fish in a range of sizes were collected to provide a representative sample of the population structure at each site (Table 1) and to establish whether there was a relationship between fish size and element concentrations in otoliths and scales.

Fish were caught by electrofishing using an InteliSYS Fish Magnet Mobile 11 (FMM) back-pack and killed using an approved U.K. Home Office Schedule 1 method. On site, fish fork length was measured and scale samples were removed from above the lateral line, slightly posterior to the dorsal fin, on the left side of each fish and stored in manila envelopes. Fish were stored at -24°C prior to removal of otoliths after partial thawing.

On the 5th - 6th of September 2007, to coincide with near base-flow conditions (Veinott & Porter, 2005) for that summer, triplicate samples of 100ml of stream water were collected at each site (Fig. 1). Stream water was syringed into acid washed polypropylene bottles through a 0.45µm membrane, then acidified with

0.5ml of 70% ultrapure nitric acid (OPTIMA, Seastar Chemicals Inc.) and refrigerated at 4°C. Powder-free vinyl gloves were worn during water sampling.

Sample preparation and analytical methodologies

To avoid procedural differences interfering with the estimation of element concentrations in fish-structures, similar sample preparation and analyses protocols, based on those outlined by Wells *et al.*, (2003a), were used for both scales and otoliths. The left-side otolith and one non-regenerated scale from each fish were manually cleaned under a dissecting microscope using nylon brushes and plastic tipped forceps. These implements were acid-rinsed in 10% nitric acid (Trace Grade, Seastar Chemicals Inc.) between uses. Samples were ultrasonically cleaned for 4min in trace element grade 3% hydrogen peroxide (Trace Grade, Fluka), triple-rinsed with 18.2MΩ water (Milli-Q) and dried overnight in a laminar flow hood. All equipment in contact with water, scale and otolith samples was new for this experiment, non-metallic, acid washed in 10% nitric acid (TraceMetal Grade, Seastar Chemicals Inc.), triple rinsed in 18.2MΩ water, dried in a laminar low hood and stored in sealed plastic bags prior to use.

Otoliths and scales were analysed in separate batches. Samples were randomised to create blocks of 6 samples with each block containing one randomly chosen sample from each site. Otoliths were glued to glass slides using epoxy resin (Araldite) (Proctor & Thresher, 1998), with the sulcus face upwards. Scales were mounted on glass slides using double sided tape (No More Nails Tape) basal plate down, so that the hydroxyapatite layer was exposed. The epoxy resin and the tape were analysed by LA-ICP-MS to confirm that the elements of interest were low or below detectable levels (Appendix E). Mounted samples were stored in air-tight containers until analysis.

Scales and otoliths were analysed with a 193nm ArF Geolas Q Plus exciplex laser ablation facility coupled to an Agilent 7500c Quadrupole Inductively Coupled Plasma Mass Spectrometer (LA-ICP-MS). During scale analysis, the laser was set to a 100 pulse count and fired at a rate of 5Hz. The energy of the laser was 6 J/cm^2. The focus of the laser could be controlled to an accuracy of 1μm which allowed the scale hydroxyapatite layer to be removed from the basal plate. The ICP-MS was set to an integration time of 10ms for each element. Blanks were measured before every 3rd sample by running the ICP-MS for 20s prior to laser ablation. During otolith analysis

the same LA-ICP-MS settings were used except that the laser pulse count was changed to 200 and the energy of the laser to 8 J/cm^2, on account of the more robust aragonite of otoliths. During trials it was established that the following isotopes were detectable in at least one of the structures: ^{7}Li, ^{24}Mg, ^{28}Si, ^{55}Mn, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{138}Ba, ^{208}Pb, and ^{238}U and so these were included in the final analysis, in addition to ^{44}Ca. Three replicate spot ablations, each 122μm in diameter, were carried out on the distal (outer) edge of each scale and otolith, to thoroughly sample the structure chemistry corresponding to the most recent (summer 2007) growth which represented the scale and otolith material most likely to have been formed at or close to the site of capture (Hamer & Jenkins, 2007). The 122μm laser spot size was considered suitable for reducing the effect of any micro-scale heterogeneity in element concentrations between structures and also for providing sufficient coverage to take account of potential differences in the incorporation rate of element impurities into the two structures (Elsdon & Gillanders, 2005b). The positions of the replicate ablations were standardised to the same growth axis within both scales and otoliths, to avoid endogenous differences in accretion rates interfering with the estimated element concentrations of the structures (Hamer & Jenkins, 2007). NIST Standard Reference Material (SRM) 610 was used to calibrate the ICP-MS facility and NIST 610 and 612 were analysed as 'unknowns' three times during each day of analysis. Examination of the NIST 610 and 612 data revealed no systematic instrument drift.

Water samples were analysed using a VG Elemental PlasmaQuad II+ ICP-MS. Three external multi-element standards and an internal standard of Ru were used in the analysis. Duplicate analyses were carried out on each sample. Duplicate analyses and triplicate samples were averaged to get a single element concentration in stream water for each site.

To test the stability of element concentrations in scale hydroxyapatite, data on the element concentrations of the freshwater growth bands of adult sea trout scales from the Dee are compared with the element concentrations in scales of the freshwater-resident *Salmo trutta* from the Dee. Similarly, data on element concentrations in the freshwater growth bands of adult sea trout scales and freshwater smolt scales from the Nith catchment in Scotland are compared. These samples were analysed as part of a separate study which was carried out on a different LA-ICP-MS to the one used in this study. While values will probably differ

due to inter-facility differences, a comparison of sea trout scale samples which were analysed on *both* LA-ICP-MS facilities, revealed a close agreement between them (Table 2).

LA-ICP-MS data processing

Scale and otolith LA-ICP-MS data were processed using the SILLS Project software (Mineralogical Association of Canada Short Course 40, Vancouver, B.C.). A plot of counts per second (CPS) for isotopes recorded during each ICP-MS run was used to isolate element concentrations corresponding to sample material removed during each ablation. The first couple of laser shots of each ablation were discarded to avoid potential surface contamination (Brophy *et al.*, 2003) and the CPS for each ablation were averaged for each element. Raw data were blank corrected. The Limit of Detection (LOD) for each element was calculated as the blank value plus three standard deviations (SDs) of the blank (Miller & Miller, 1993). Ca was used as an internal standard as it is a major component of hydroxyapatite and aragonite and its concentration typically varies <1% in both structures (Clarke *et al.*, 2007). The concentration of Ca in otoliths and scales was determined from the stoichiometry of aragonite to be 400,000μg/g (Milton & Chenery 2001a). Concentrations of the remaining elements in the analysis were estimated in relation to this and reported in μg/g of aragonite (Milton & Chenery 2001a). Hydroxyapatite is not a stoichiometric crystal and so the value of 374,000 μg/g (±4,000μg/g) was used for the Ca content of scale hydroxyapatite as reported in a previous study (Flem *et al.*, 2005).

Analytical precision during the scale analysis was calculated as the relative standard deviation (RSD) of element:Ca in the NIST SRMs which had been analysed as 'unknowns'. Precision for the NIST SRM 610 and 612 averaged across the scale and otolith analyses were as follows (shown as element followed by RSD (%) for NIST 610/612): Li 3/5, Mg 3/3, Si 3/3, Mn 4/4, Co 2/4, Cu 5/5, Zn 14/12, Sr 5/4, Ba 7/7, Pb 9/6 and U 9/7. No systematic drift was detected during the analysis.

Average recovery of elements in the NIST 610/612 during the scale and otolith analysis, based on published element concentrations in these SRMs (Pearce *et al.*, 1997), were as follows (shown as percentage recovery of each element for NIST 610/612): Li 100/106, Mg 99/81, Mn 99/101, Co 100/104, Cu 100/112, Zn 101/114,

Sr 100/102, Ba 100/102, Pb 100/105 and U 100/108 (no published values for Si concentration in the NIST SRMs were available).

Where ablation data for an element fell below the LOD, an element concentration for a given sample was derived from the remaining replicate ablations, providing that the element's concentration was above the LOD for at least one ablation. The percentages of otolith samples for which all three replicate ablations fell below the LOD for a given element were as follows: Cu 2%, Pb 4%, Li 63%, and U 92%. Li, and U data were therefore excluded from further statistical analysis. The percentages of scale samples for which all three replicate ablations fell below the LOD for a given element were: Li 4.5%. Occasionally, data that fell below the LOD were negative after blank correction in which case a positive constant, corresponding to the largest negative value plus 1, was added to the data to allow $\log_{10}$ transformation (Fowler *et al.*, 1995).

The data for the three ablations were averaged to provide one concentration for each element in each scale or otolith. Outliers were identified as values greater than five standard deviations away from the mean concentration of that element, pooled across all sites (Veinott & Porter, 2005). Less than 2% of the data for any element were classed as outliers and these were replaced with the mean value of that element, pooled across all sites. Element concentrations were $\log_{10}$ transformed prior to statistical analysis (Muhlfeld *et al.*, 2005) to meet assumptions of homoscedasity (tested with Levene's test) for parametric tests. Despite transformation, element concentration data failed normality tests (tested with Anderson-Darling's test), however, graphical inspection of the data (McGuiness, 2002), revealed only minor deviations from normality.

Statistical analysis

Element concentrations in calcified structures have been shown to vary with fish length/otolith size (Thorrold *et al.*, 1998; Brophy *et al.*, 2003). In this study, fish fork length was used to examine the effect of fish size on element concentrations in fish structures. ANCOVA was used to reveal common within-group regression slopes of element concentration and fork length at each site by using each element in the scales/otoliths as the response variable, site as a category variable and fork length as a covariate. Providing that the assumption of equal slopes across sites was met, the effect of fork length on element concentrations was removed by using the

common within-group regression coefficient in the following equation, adapted here from Almeida *et al.*, (2008):

$$AC_{ij} = UAC_{ij} - [\beta \times (Log_{10}FL_j - Log_{10}\underline{FL})];$$

where AC_{ij} is the adjusted transformed element concentration for element *i* of the *j* specimen, UAC_{ij} is the unadjusted character measurement for element *i* of the *j* specimen (UAC was log_{10} transformed), β is the equal common within-group regression coefficient of element *i* (log_{10} transformed) regressed against fork length (log_{10} transformed), FL_j is the fork length of the *j* specimen (log_{10} transformed) and $\underline{FL}$ is the mean fork length of all fish (log_{10} transformed).

The ANCOVA was repeated (this time with the adjusted element concentration data as the response variable) to confirm that the regression slope between fork length and element concentration was no longer significant ($P>0.05$). Within-site regressions of adjusted element concentrations were used to confirm that length was no longer a significant covariate. The adjusted element data were used in all subsequent analyses (except when correlating element concentrations in otoliths and scales from the same fish). ANCOVA is robust to departures from normality providing data are homoscedastic (Olejnik & Algina, 1984) and it has been used to correct the effects of size on non-normal data (Claytor *et al.*, 1991; Almeida *et al.*, 2008).

Scatter plots and correlations were used to examine the relationship between mean element concentrations in scales and otoliths at each site and element concentration in stream water (Pearson's Product Moment Correlation Coefficient was used where data met assumptions of normality and Spearman's Rank Correlation Coefficient where not, denoted r_p and r_s respectively). Correlations were conducted with stream water data expressed as absolute concentrations (log_{10} transformed) and as element:Ca ratios (log_{10} transformed). Partition coefficients (D_{Me}) (Morse & Bender, 1990), the ratio of element:Ca in otoliths/scales, to element:Ca in water, were calculated for those element concentrations in scales/otoliths which correlated with stream water. A partition coefficient of 1 would mean that the element:Ca ratio in the calcified structure matches the element:Ca ratio in the surrounding water. This provided a standardised way of comparing the degree

of incorporation of elements in each of the structures in relation to element concentrations in water (Elsdon & Gillanders, 2003).

One-way ANOVA and Scheffe's post-hoc comparisons were used to determine whether there were significant differences in element concentrations among sites. Non-normality does not preclude the use of ANOVA, providing data are homoscedastic (McGuinness, 2002). The accuracy with which fish could be classified to their site of origin based on their scale and otolith chemistry was compared using Linear Discriminant Function Analysis (LDFA) (Wells *et al.*, 2000b; Clarke *et al.*, 2007). The LDFA involved an 'original' classification (which used the discriminant functions to classify the same samples that were used to develop the functions) and a 'cross-validation' (CV) classification (which involves leaving one sample out of the dataset before establishing the discriminant functions and then classifying the sample that was removed) (Sharma, 1996). Minor deviations from normality do not preclude the use of LDFA (Leakey *et al.*, 2008). However, a cautionary approach was adopted and non-parametric Multinomial Logistic Regression (MLR), was used to validate the overall and group classifications achieved with the LDFA and to monitor potential effects of non-normality on the LDFA results. Multicollinearity was detected among elements and therefore a stepwise LDFA was avoided and non-stepwise LDFA Standardised Discriminant Function Coefficients were used to indicate the relative importance of element concentrations in the LDFA classification (Sharma, 1996). Canonical Variate Plots provide a visual representation of the classification of individual fish to their site of origin. Cohen's Kappa statistic was used to compute the chance-corrected agreement between actual and predicted group (site) memberships of fish (Titus *et al.*, 1984; Barnett-Johnson *et al.*, 2008). The Kappa statistic ranges between 0 (which would indicate the classification to site was no improvement over that achieved by chance) and 1 (which would indicate that there was perfect agreement in the classification to site when taking into account classification by chance).

The relationship between paired element concentrations in scales and otoliths of individual fish was examined using scatter plots and correlations (Wells *et al.*, 2000b; Gillanders, 2001).

Differences in the element composition of the freshwater growth bands of marine *Salmo trutta* (sea trout) and resident *Salmo trutta* in the Dee and Nith

catchments were tested using One-way ANOVA where data met assumptions of homogeneity and Kruskal-Wallis where not.

All statistical analysis was carried out in SPSS (v. 16) and MINITAB (v. 14).

Results

Site locations and fork length of *Salmo trutta* collected in the Dee catchment in 2007 are shown in Figure 1 and Table 1 respectively.

Relationship between scale, otolith and stream water chemistry

Element concentrations were generally between 1-3 orders of magnitude higher in scales than in otoliths (Fig. 2, Table 3) except for Sr which had similar mean concentrations among sites, ranging between 189 – 386 μg/g and 213 – 539 μg/g in scales and otoliths respectively. Paired scale and otolith element concentrations in individual fish were positively correlated for Mn, Sr, Ba and Pb (Fig. 2). The significant correlation for Pb was caused by high values of Pb in both structures at site AU. When AU was excluded, the relationship was no longer significant (r_s=0.16; df=4; P=0.62). No significant correlations were found between Co, Mg, Cu, Zn and Si in otoliths and scales (P>0.05)(Fig. 2).

The correlations between element:Ca in stream water at individual sites and average element concentrations in scales were very strong between Mn in scales and Mn:Ca in stream water and between Ba in scales and absolute concentrations of Ba in stream water (Fig. 3). In otoliths, there were positive correlations between Mg in otoliths and Mg:Ca in stream water, Mn in the otoliths and Mn:Ca in the stream water and between Ba in the otoliths and absolute concentrations of Ba in the stream water (Fig. 3). No significant correlations were found for the remaining elements (P>0.05) (N.B. no data on Li and Si concentrations in stream water were available).

To compare the concentrations of elements in otoliths/scales with concentrations in stream water, partition coefficients (D_{Mc}) were calculated for those elements which correlated with water chemistry (Fig. 3, Table 4).

Variability in element concentrations in scales and otoliths among 6 sites in the Dee catchment

Significant differences in fish fork length were found in *Salmo trutta* among the 6 study sites (ANOVA: $F_{5,83} = 2.70$; $P = 0.026$). Statistically significant common within-group regression slopes were found for $\log_{10}$Si (β= -0.85; P<0.001), and $\log_{10}$Zn (β= -0.40; P=0.012) in scales and $\log_{10}$Mg (β= -0.38; P=0.018) and $\log_{10}$Ba (β= -0.32; P=0.019) in otoliths. The effect of length on element concentrations was removed and the adjusted data used in all remaining statistical analyses.

There were significant differences in $\log_{10}$ element concentrations among sites for 6 elements in otoliths and 11 elements in scales (Table 5). Intra-site variability in element concentrations was generally higher in scales than in otoliths (as indicated by higher F ratios for otoliths) except for Sr and Pb (Table 5). For instance, although concentrations of Mn were positively correlated between scales and otoliths, there were fewer pairs of sites between which there were significant differences in Mn concentrations in scales compared with otoliths (Table 5). Also, the ANOVA F ratio for Mn in scales was much lower than in otoliths, suggesting that intra-site variability in scale chemistry was higher than inter-site variability when compared with otoliths (Table 5).

Sr, Mn and Ba were the elements that exhibited the most variation in concentrations among sites, in both scales and otoliths (Scheffe's post-hoc tests; Table 5). The same pairs of sites showed significant differences in Sr in both structures (Table 5). Significant differences in concentrations of Co in scales were detected among sites paired with CW, which exhibited Co concentrations approximately x5 higher than at other sites (Table 3, Fig. 2).

Classification of fish to site of origin based on scale and otolith chemistry

89% of otoliths and 88% of scales were correctly classified to their site of origin using cross-validation (CV) LDFA on all element concentrations which differed significantly among sites (Table 5). The standardised canonical discriminant function coefficients indicate that Sr and Mn were the most important variables in the discrimination for otoliths and Sr and Ba were the most important variables in the discrimination for scales, as these elements had the highest absolute values in the first function, which explained a high proportion of the variance (Table 6). The

chance corrected CV LDFA classification success for all elements using Cohen's Kappa statistic was 0.87 (±0.6 CIs) for both scales and otoliths.

The LDFAs were repeated, this time limiting the elements to those which were most important in the previous LDFA and which are routinely used in studies of this nature (Sr, Mn, Ba and Mg) (Wells *et al.*, 2000b; Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005). In both structures Sr, Mn and Ba showed the highest number of significant differences between pairs of sites (Table 5), indicating that these elements might be useful in discriminating site of origin. Despite limiting the number of elements in the analysis, the CV classification accuracy for otoliths was still 89% and only dropped to 86% for scales. The Cohen's Kappa statistic was 0.87 (±0.6 CIs) in otoliths and 0.84 (±0.6 CIs) in scales based on using Sr, Mn, Ba and Mg data only in the LDFA.

Canonical Variate Plots indicate that fish from the 6 sites roughly separate into 3 groups based on concentrations of Sr, Mn, Ba and Mg in both otoliths (Fig. 4) and scales (Fig. 5). CW and LF site cluster together, as do AB and CR, and AU and SB. These groups loosely correspond to sites in the upper, middle and lower regions of the catchment respectively (Fig. 1). Fish which were misclassified to their site of origin, tended to be allocated to the remaining site in the same region (Table 7). This is probably due to increasing concentrations of Mn in structures at sites from the lower to the upper regions of the catchment and relatively high concentrations of Sr in structures from sites in the middle region (Table 3).

The LDFAs were repeated for a 3rd time, using all elements in scales which showed significant differences in element concentrations among sites, excluding Sr, Mn, Ba and Mg (i.e. Zn, Pb, Co, Cu, Si, Li and U) . Despite removing these elements from the LDFA, 68% of fish could be classified to their site of origin using CV LDFA.

To monitor the effects of minor deviations from normality in the data on the LDFA results, comparisons were made between the 'original' (i.e. non-cross validated) classification accuracies achieved using LDFA and MLR for scales and otoliths (Table 8). MLR resulted in a higher overall classification accuracy for both structures when compared with the 'original' LDFA, with classification accuracies of 96% and 92% for otoliths and scales respectively when including data for Sr, Mn, Ba and Mg only. Classification accuracy of fish at each of the 6 sites using LDFA and MLR was also similar (Table 8).

Relationship between scale chemistry of freshwater-resident and sea run Salmo trutta

There appears to be some degree of post-depositional change in *Salmo trutta* scale chemistry during marine residency for sea trout originally from the Dee (Table 3) and the Nith (Table 9) catchments. For instance, concentrations of Sr and Mg were significantly higher in the freshwater growth bands of Dee sea trout scales compared with freshwater-resident fish scales from the same catchment (Table 3). This pattern was repeated in the sea trout and freshwater smolt scales from the Nith catchment (Table 9). While this does not specifically address whether post-depositional change occurs in *fresh water*, it does indicate that it might be a possibility.

Discussion

The results of this study have shown that there are significant variations in element concentrations in recently formed *Salmo trutta* scale hydroxyapatite and otolith aragonite among sites in the Dee, a small upland catchment (~1800km^2). In addition, the regional differences in element concentrations reported in both scales and otoliths in this study suggest that the origins and movement patterns of *Salmo trutta* might potentially be distinguishable between the upper, middle and lower regions of the Dee catchment. The high classification accuracies at the 6 study sites (>7.5km between neighbouring sites, Fig. 1) suggest that relatively fine-scale movement patterns of fish might be distinguishable in the catchment through the use of chemical tags in scales or otoliths. It is likely that the heterogeneity in the bedrock geology and stream water chemistry observed in the Dee catchment (BGS, 1999), contributes to the wide ranging values in structure chemistry observed over relatively small spatial scales.

The general opinion in the literature is that otoliths provide a superior biogeochemical tag to non-lethally collectable alternatives, such as scales (Wells *et al.*, 2003b; Clarke *et al.*, 2007). The findings of this study suggest that scales may offer a comparable account of spatial variations of some elements (e.g. Sr), in recently deposited scale hydroxyapatite. In this study, almost twice as many element

impurities were found to be significantly different among sites in scales compared with otoliths. However, the usefulness of these additional elements in the LDFA was limited, resulting in a drop in classification error of only 2% when all elements were used in the LDFA. However, when Zn, Pb, Co, Cu, Si, Li and U in scales were considered in isolation (i.e. without Sr, Mg, Mn and Ba) the CV classification accuracy was 68% suggesting that these elements could be important biogeochemical tags, possibly if more sites were included in the analysis (Gillanders, 2005). In this study, LDFA appears to offer a more conservative estimate of 'original' classification accuracy when compared with the non-parametric alternative, MLR (as has been documented elsewhere; Pragar & Fabrizio, 1990).

In this study, similar classification accuracy was observed for *Salmo trutta* collected from 6 sites in the Dee catchment based on scale and otolith chemistry. The poorer classification of scales compared with otoliths reported in previous studies has been attributed to higher variability in element concentrations in scales (Wells *et al.*, 2003a). This was also the case for Mn, Ba and Mg in this study but not for Sr. Sr was one of the most important elements in classifying the origin of fish in this study, as has been found in previous studies in freshwater (Wells *et al.*, 2003a; Muhlfeld *et al.*, 2005). Scales and otoliths provided a similar record of variability in Sr among sites in the Dee, as demonstrated by the strong correlations between Sr in scales and otoliths, and by the identical pairs of sites between which significant differences in Sr concentration in both structures were found. The high ANOVA F ratio for Sr in scales indicates that, in contrast to other studies, this structure might even be superior to otoliths as a record of Sr variability in *Salmo trutta* structures in the Dee (Wells *et al.*, 2003a; Clarke *et al.*, 2007).

Both laboratory and field-based studies have reported a correlation between concentrations of either Sr, Mg, Mn and Ba in water and their corresponding concentrations in fish structures (Wells *et al.*, 2000a; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007), but the relationships can be inconsistent between studies/species (Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007) and between structures of the same fish (Clarke *et al.*, 2007). Ambient element concentrations in water have been more tightly correlated with otoliths compared with scales (Wells *et al.*, 2000a). One of the more consistent relationships reported in the literature is between Sr in fish-structures and water, although this relationship is not always identifiable in respect of scales (Clarke *et al.*, 2007). In this study the relationship was not statistically

significant, however, in a subsequent study on scale chemistry in the Dee, it was found to be significant when the number of study sites was increased from 6 to 12 (Chapter 4).

In this study, the concentrations of some elements in either scales or otoliths, were uncorrelated with their concentrations in stream water, although such a relationship has been presented in other studies (e.g. Cu in Milton & Chenery 2001b). While this might reflect the fact that water samples were collected on one date only (and might therefore not be representative of concentrations of all elements at each site), regulation of some elements which are biologically important (e.g. Zn) or toxic (e.g. Pb) might be expected (Gibson-Reinemer *et al.*, 2009). Irrespective of whether element concentrations are correlated with water, they might still provide a viable tag if their concentrations in scales differ consistently between geographical locations (Gillanders, 2005). In this study, elements such as Cu and Co in scales do vary in concentration among sites and could become more important in the discrimination of geographical origins of fish, if the number of sites among which fish had to be classified were increased.

The partition coefficients were highest for manganese (D_{Mn}) in scales indicating that there was a greater incorporation of Mn in scales compared with the remaining elements in both structures in relation to element:Ca in water. Despite the limited incorporation of Mn in otoliths compared with scales, this structure performed better than Mn in scales, as a biogeochemical tag. Intra-site variability in Mn in both scales and otoliths increased with increasing Mn concentration in this study (Fig. 2), a trend which has been reported elsewhere for Ba and Sr (Elsdon & Gillanders, 2003). Interestingly, a relationship was found between absolute concentrations of Ba in water and Ba:Ca in scales and otoliths. A similar relationship has been reported elsewhere in a study on Ba in otoliths (Martin & Thorrold, 2005). This relationship has been attributed to an inhibition of Ca uptake by Ba at some point before deposition in the calcified structure takes place (Martin & Thorrold, 2005).

The findings to date indicate that elements in biological structures in freshwater fish are derived primarily from water rather than the diet (Milton & Chenery 2001b; Brown & Severin, 2009). Changes in the incorporation rate of elements may occur due to physiological differences between fish (Campana, 1999) or with changes in the calcified structure composition of scales after initial formation

(Fouda, 1979). While *Salmo trutta* scales can suffer resorption, this generally occurs during migrations of sea trout to their freshwater spawning grounds (Elliott & Chambers, 1996), rather than in freshwater-resident individuals. Resorped scales are distinguishable under a dissecting microscope (Elliott & Chambers, 1996) and can be avoided if necessary. In the present study, for key discriminating elements such as Sr and Ba, the composition of the freshwater growth bands in the scales of sea trout caught on return to the Dee and Nith catchments, were significantly different from their freshwater-resident counterparts. The differences in element concentrations were indicative of exposure to element concentrations in the marine environment and suggests that the hydroxyapatite of *Salmo trutta* scales might undergo continued crystallisation or reworking once formed as suggested by Fouda (1979) for the common goby, *Pomatoschistus microps*. Concentrations of Sr in the marine environment are generally higher than in freshwater (Brown & Severin, 2009) and the range of values in the freshwater growth bands of sea trout scales were higher when compared with the scales of fish which had only resided in freshwater (Table 3 and 9). Ba concentrations are generally higher in freshwater than salt-water (Elsdon & Gillanders, 2005a) and the concentrations of this element in the freshwater growth bands of smolts from the Nith were generally higher in range than in sea trout from the same catchment although this pattern was less evident in the Dee catchment. However, despite the apparent potential for post-depositional change in hydroxyapatite, the pilot study showed that there were significant differences in element concentrations between the freshwater and marine hydroxyapatite in sea-run *Salmo trutta* scales (see Introduction and Appendix C), indicating that reworking of the freshwater growth bands is not complete. Given that element concentrations can vary widely between freshwater and marine environments, this is a fairly severe test of post-depositional change in *Salmo trutta* scale chemistry. It is possible that post-depositional change could be less pronounced in fish residing in freshwater and scales might therefore be sufficiently stable to provide an alternative structure to otoliths for determining natal origins and movement patterns of freshwater-resident *Salmo trutta*. Despite the potential for post-depositional changes in scale chemistry, the element composition of scales has been sufficiently stable to reveal movement patterns and origins of fish (Coutant & Chen, 1993; Pender & Griffin, 1996; Adey *et al.*, 2009).

A key consideration when comparing the use of biological structures as biogeochemical tags is whether the tag is temporally stable on a seasonal and inter-annual basis. Temporal changes in baseline element concentrations among locations can alter the biogeochemical tag of structures at a given site (Wells *et al.*, 2000b). The year in which the hydroxyapatite was formed in the sea-run and freshwater-resident fish in this study would have differed and so it is possible that the difference in the range of element concentrations could be due to changes in the baseline element signatures among years. However, spatial variability in element concentrations in stream water in Wales is thought to vary in range so widely among geographical locations that patterns of spatial variability in stream water chemistry in the region are relatively robust to inter-annual changes (BGS, 1999).

The intended application will have a significant bearing on the suitability of fish structures as biogeochemical tags. Otoliths, with their daily growth bands which start forming prior to hatching (Campana & Neilson, 1985), provide a record of element impurities which is superior in temporal resolution when compared with scales which form at some point after hatching and rarely have distinguishable daily growth bands. However, this does not prohibit the application of scales as biogeochemical tags, providing that a relatively coarse temporal resolution is acceptable (e.g. Adey *et al.*, 2009). This study has shown that there is some evidence of regional patterning in element impurities in recently deposited scale hydroxyapatite and otolith aragonite of *Salmo trutta* from the Dee catchment. This will be explored in a subsequent study designed to increase the spatial coverage of sites in the catchment which should further elucidate the applicability of structure chemistry as a fisheries management tool for identifying fish to site of origin in the Dee catchment. Scales might offer a non-lethal biogeochemical tag, comparable in performance to otoliths. However, further work needs to examine the degree of post-depositional change in scale hydroxyapatite. This would probably require aquaria-based studies in which the effect on scale chemistry of exposing fish to changing element concentrations in water and diet could be monitored.

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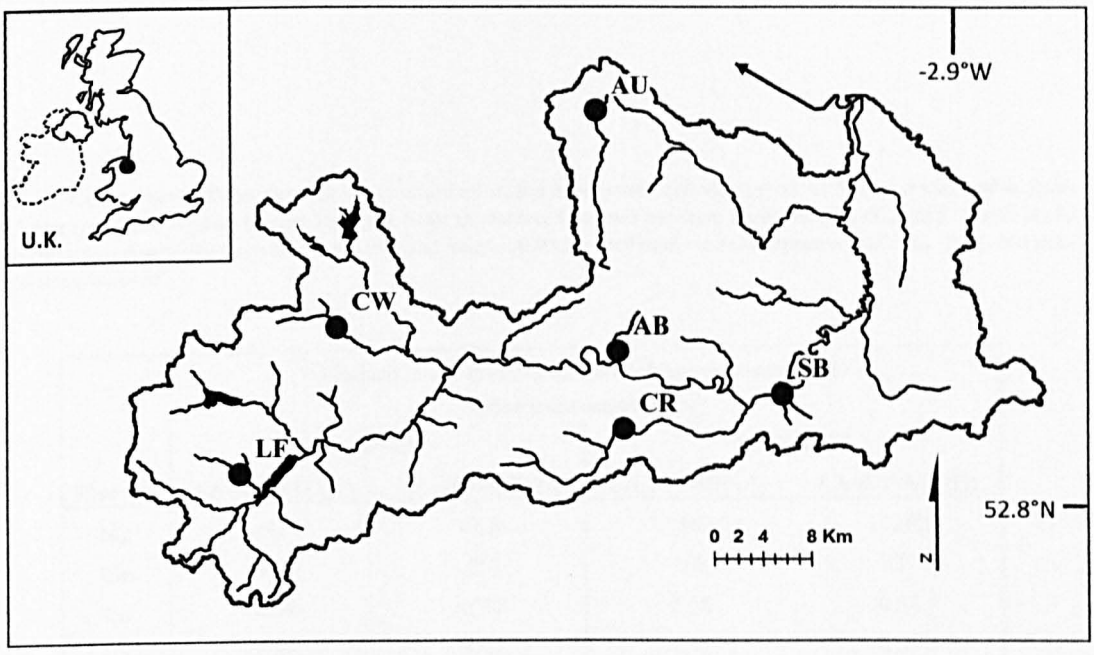

Figure 1. Location of the Dee catchment showing the catchment boundary and main river network (river discharge point into the Dee estuary is shown with a black arrow). 2007 electrofishing survey sites (black circles) and corresponding site codes AB, AU, CW, CR, LF and SB described in Table 1, are also shown.

Table 1. Dee electrofishing site locations [British National Grid Reference (NGR)], sample sizes (n) and details on the fork lengths (FL) of *Salmo trutta* collected from 6 sites in the Dee river catchment.

Site	Site Code	NGR	n	Mean FL (±SE) (mm)	FL Range (mm)
Abbey Brook	AB	SJ206444	11	140 (±5)	113-181
Alyn Upstream	AU	SJ188653	14	187 (±13)	132-258
Ceirw	CW	SH962465	16	153 (±11)	83-250
Ceiriog	CR	SJ211377	13	143 (±8)	70-198
Llafer	LF	SH877338	15	148 (±10)	86-257
Shellbrook	SB	SJ348407	14	168 (±9)	109-222

Table 2. Comparison of the element concentrations in the freshwater growth region of *Salmo trutta* scales from the Luce (sea trout A) and Border Esk (sea trout B) detected by two separate facilities (LA-ICP-MS A and B) to determine the similarity between scale element concentrations analysed on two separate facilities. N.B. No data on Si are available.

	Element concentrations in the freshwater growth bands of Sea trout scales (μg/g)			
	Sea Trout A		Sea Trout B	
Element	LA-ICP-MS (A)	LA-ICP-MS (B)	LA-ICP-MS (A)	LA-ICP-MS (B)
Mg	4313	4720	9563	11392
Mn	120	156	199	327
Co	0.38	0.37	0.28	0.67
Cu	2.51	3.98	1.56	2.56
Zn	862	906	530	457
Sr	1097	1150	1127	1465
Ba	8	10	20	27
Pb	203	215	1.4	2
U	0.15	0.16	0.04	0.05
Li	3.28	4.31	8.44	12.47

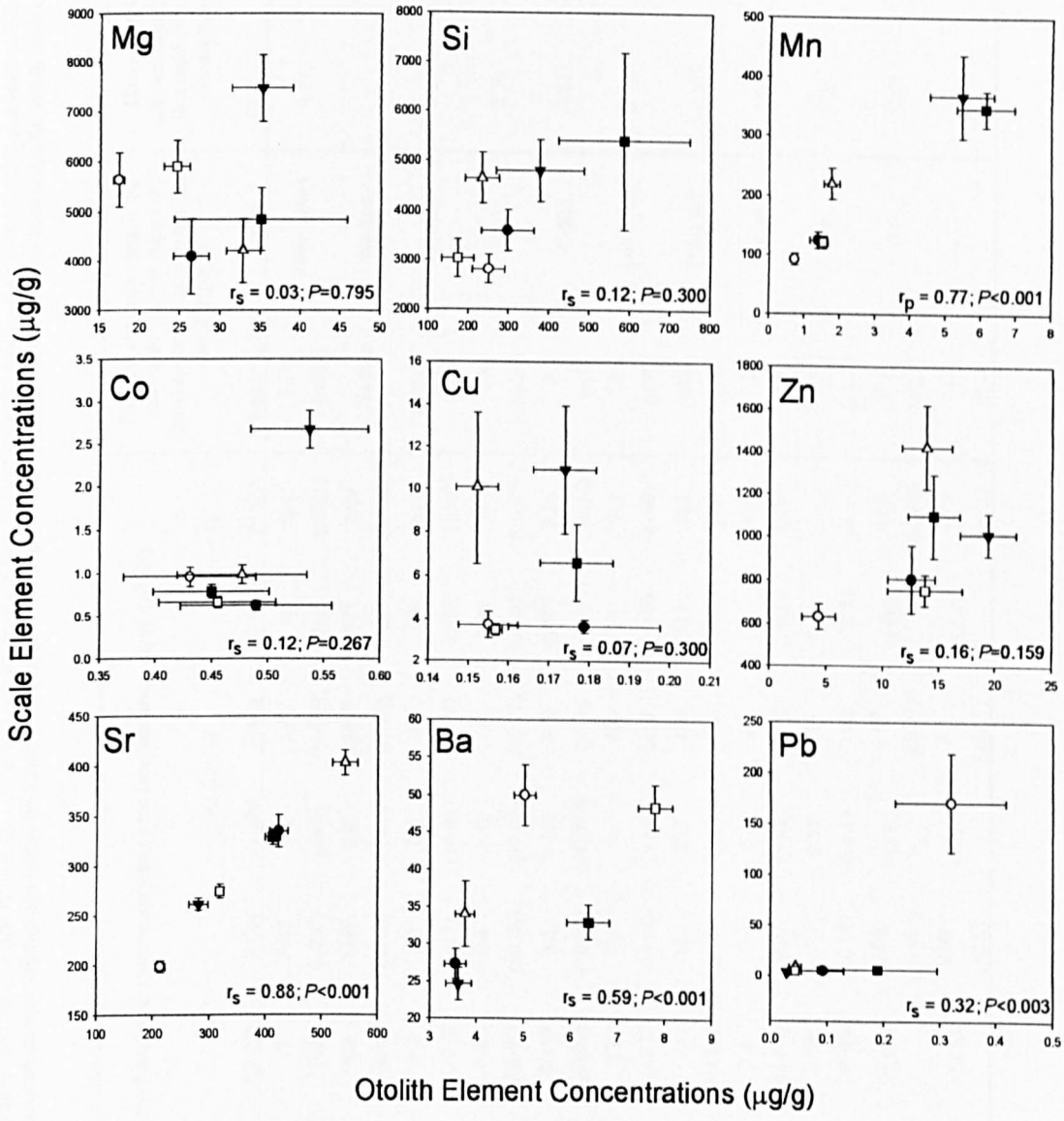

Figure 2. Scatter plots of mean elemental concentrations (µg/g ± 1SE) in scales and otoliths at 6 sites in the Dee catchment (AB, closed circles; AU, open circles; CW, closed triangles; CR, open triangles; LF closed squares and SB, open squares). Correlations using Spearman's Rank Correlation Coefficient (r_s) or Pearson's Product Correlation Coefficient (r_p) between element concentrations in paired scales and otoliths from the same fish (n=83) are also shown.

Table 3. Mean (±1SE) element concentrations in otoliths and scales of *Salmo trutta* from 6 sites in the Dee catchment (unadjusted for the effect of fork length on element concentrations (see Results section) to allow a direct comparison of element concentrations between scales and otoliths to be made) and One-way ANOVA results on element concentrations in Dee resident scales [pooled across all sites (n=83)] and sea trout (n=27) . *Denotes Kruskal-Wallis test rather than ANOVA test. Elements in otoliths/scales which were consistently below the Limit of Detection are denoted '<LOD'. Elements in the adult sea trout scales for which some of the data are below the LOD are shown in italics.

	Mean element concentrations in otoliths and scales of freshwater-resident *Salmo trutta* at six sites in the Dee catchment (µg/g) (±1SE)												Element concentrations in the freshwater growth region of adult sea trout scales in the Dee (n=27) (µg/g)		One-way ANOVA on element concentrations in Dee resident trout (n=83) and sea trout (n=27)	
	LF (n=15)		CW (n=16)		CR (n=13)		AB (n=11)		AU (n=14)		SB (n=14)					
Element	Otoliths	Scales	Otoliths	Scales	Otoliths	Scales	Otoliths	Scales	Otoliths	Scales	Otoliths	Scales	Mean (±1SE)	Range	$F/H_{1,109}$	P
Mg	33 (±10)	4636 (±608)	35 (±4)	7152 (±665)	34 (±3)	4026 (±671)	25 (±2)	3913 (±737)	18 (±0.5)	5397 (±516)	25 (±2)	5645 (±501)	6978 (±602)	1266-12604	4.91	0.029
Si	599 (±161)	5173 (±1707)	389 (±106)	4589 (±616)	223 (±40)	4452 (±487)	296 (±65)	3446 (±400)	250 (±40)	2695 (±282)	173 (±40)	2904 (±374)	No data	No data	-	-
Mn	6.1 (±0.8)	330 (±29)	5.4 (±0.9)	351 (±70)	1.8 (±0.2)	209 (±24)	1.4 (±0.2)	119 (±13)	0.7 (±0.06)	88 (±9.4)	1.5 (±0.1)	116 (±10)	202 (±21)	36-460	0.05	0.826
Co	0.45 (±0.05)	0.76 (±0.07)	0.54 (±0.05)	2.5 (±0.22)	0.48 (±0.05)	0.95 (±0.10)	0.49 (±0.07)	0.6 (±0.06)	0.43 (±0.06)	1 (±0.10)	0.46 (±0.05)	0.64 (±0.07)	0.8 (±0.4)	0-10	2.76	0.1
Cu	0.177 (±0.009)	6.3 (±1.68)	0.173 (±0.008)	10.45 (±3)	0.169 (±0.018)	9.6 (±3.39)	0.179 (±0.019)	3.5 (±0.30)	0.155 (±0.007)	3.56 (±0.6)	0.157 (±0.017)	3.34 (±0.26)	6 (±3)	0-80	4.31	0.04
Zn	15 (±2.3)	1049 (±189)	19 (±2.6)	964 (±97)	16 (±3.1)	1358 (±190)	13 (±2.1)	770 (±148)	4 (±1.6)	604 (±55)	14 (±3.3)	719 (±69)	524 (±45)	197-1162	20.39	<0.001
Sr	413 (±13)	315 (±7)	281 (±17)	249 (±6)	539 (±21)	386 (±11)	423 (±15)	321 (±16)	213 (±8)	189 (±6)	319 (±7)	262 (±7)	1056 (±33)	755-1515	60.57*	<0.001
Ba	6.2 (±0.4)	32 (±2)	3.6 (±0.3)	23 (±1.2)	3.8 (±0.2)	33 (±4.2)	3.4 (±0.2)	26 (±2)	5.4 (±0.3)	49 (±4)	8 (±0.3)	47 (±3)	30 (±3)	10-93	3.03	0.084
Pb	0.19 (±0.11)	6 (±1.4)	0.03 (±0.004)	3.3 (±1)	0.05 (±0.01)	7.5 (±2.4)	0.09 (±0.04)	5 (±0.54)	0.32 (±0.1)	163 (±46)	0.04 (±0.01)	5 (±1.01)	4.5 (±1.2)	1-25	6.21	0.014
U	<LOD	0.02 (±0.004)	<LOD	0.03 (±0.01)	<LOD	0.04 (±0.01)	<LOD	0.03 (±0.004)	<LOD	0.07 (±0.01)	<LOD	0.06 (±0.01)	*0.4 (±0.2)*	*0-6*	52.36	<0.001
Li	<LOD	1.73 (±0.4)	<LOD	0.73 (±0.1)	<LOD	1.27 (±0.2)	<LOD	2.99 (±0.6)	<LOD	0.3 (±0.06)	<LOD	1.55 (±0.2)	*4.48 (±1)*	*0-13*	14.69	<0.001

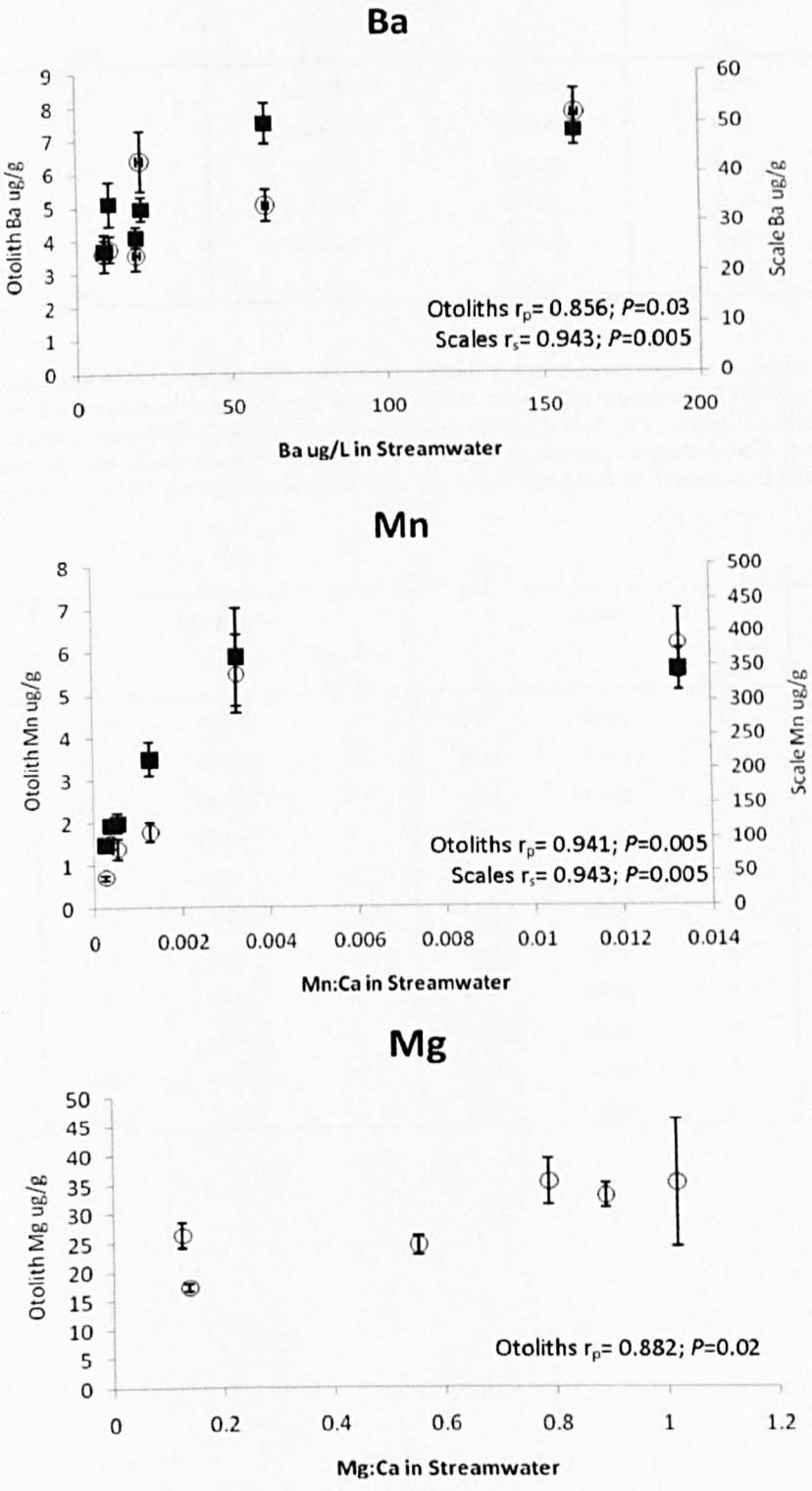

Figure 3. Scatter plots of mean (μg/g±1SE) element concentrations in otoliths (hollow circles) and scales (solid squares) and stream water. Correlation analysis of element concentrations in scales/otoliths and water are also shown.

Table 4. Mean partition coefficients (D_{Me}) of element:Ca for scales and otoliths of *Salmo trutta* at 6 sites in the Dee catchment. Partition coefficients were calculated as the ratio of element:Ca in scales/otoliths to element:Ca stream water.

	Otolith		Scales
Site	D_{Mg}	D_{Mn}	D_{Mn}
AB	0.00053	0.0064	0.59
AU	0.00032	0.0070	0.91
CW	0.00011	0.0042	0.29
CR	0.00009	0.0035	0.44
LF	0.00009	0.0012	0.07
SB	0.00011	0.0102	0.82

Table 5. One-way ANOVA results for element concentrations in otoliths and scales among sites (df = 5,82). *Column shows the number of pairs of sites between which there were significant differences ($P<0.05$) in element concentration using Scheffe's Post Hoc comparisons (out of a total of 15 pairs). † Column shows the number of pairs of sites which showed significant differences in element concentration in *both* scales and otoliths. Elements in otoliths for which >50% of data fell below the Limit of Detection (LOD) are denoted '<LOD'.

	Otolith			Scales			Matching post hoc pairs†
Element	*F*	*P*	Post hoc pairs*	*F*	*P*	Post hoc pairs*	
Sr	27.88	<0.001	13	72.37	<0.001	13	13
Mn	52.23	<0.001	10	26.24	<0.001	8	7
Ba	22.01	<0.001	8	14.16	<0.001	8	4
Zn	9.63	<0.001	5	4.21	0.002	1	1
Pb	8.36	<0.001	3	38.57	<0.001	5	3
Mg	6.64	<0.001	2	4.21	0.002	2	0
Co	0.59	0.71	0	33.8	<0.001	5	-
Cu	1.01	0.417	0	4.53	0.001	1	-
Si	1.52	0.194	0	2.8	0.022	0	-
Li	<LOD	<LOD	-	12.55	<0.001	5	-
U	<LOD	<LOD	-	8.81	<0.001	3	-

Table 6. Standardised Canonical Discriminant Function Coefficients for the LDFAs performed on otoliths and scales, using all element which exhibited significant differences in concentrations between sites in each structure (Table 5). The two most important elements in the first LDFA function are underlined and shown in bold.

	Otolith LDFA Function				Scale LDFA Function				
Element	1	2	3	4	1	2	3	4	5
Ba	-0.3	-0.01	0.94	-0.22	**1.08**	-0.37	-0.38	0.46	0.63
Mg	0.3	-0.14	-0.15	-0.53	0.76	0.88	-0.14	0.41	0.44
Mn	**0.58**	0.83	0.1	0.35	-0.56	0.96	0.3	0.73	0.07
Pb	-0.37	-0.11	0.08	0.85	0.4	-0.16	1.32	-0.02	-0.05
Sr	**0.92**	-0.44	0.17	0.22	**-1.05**	-0.62	0.34	-0.36	0.28
Zn	0.07	-0.04	-0.09	-0.23	-0.35	-0.02	-0.14	0.51	0.68
Co	-	-	-	-	0.06	0.63	0.14	-0.47	-0.02
Cu	-	-	-	-	-0.03	0.27	-0.29	-0.61	-0.42
Li	-	-	-	-	-0.16	-0.16	-0.09	0.32	-0.61
Si	-	-	-	-	-0.04	0.01	0.24	-0.11	0.11
U	-	-	-	-	0.06	-0.09	-0.89	-0.65	-0.04
Cumulative variance explained (%)	73	89	97	100	61	88	95	99	100

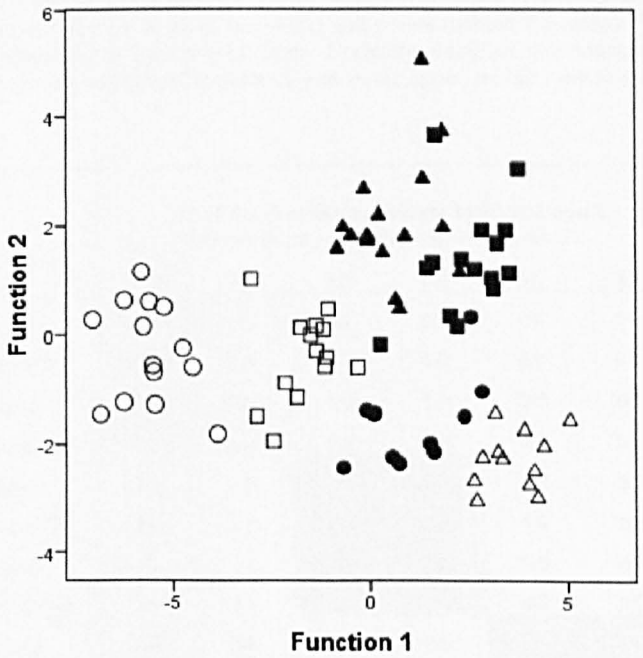

Figure 4. Canonical scores plot of the first 2 discriminant functions in the LDFA on Mn, Ba, Mg and Sr in otoliths at 6 sites in the Dee catchment (AB, closed circles; AU, open circles; CW, closed triangles; CR, open triangles; LF closed squares and SB, open squares).

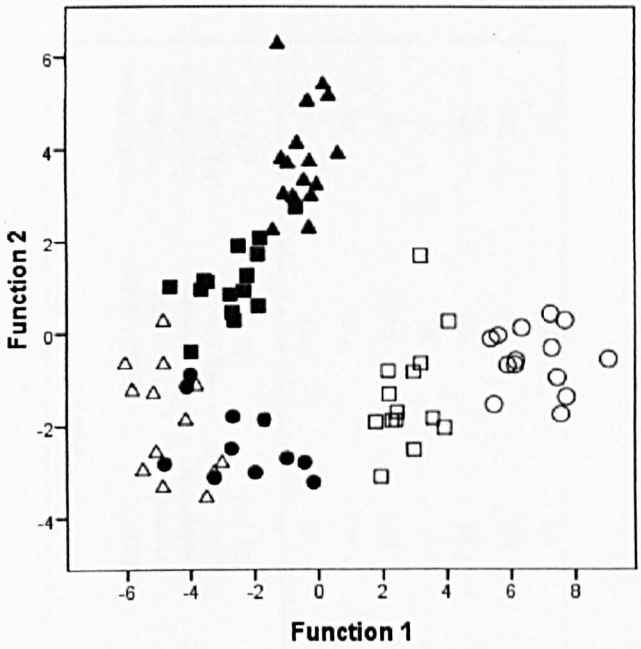

Figure 5. Canonical scores plot of the first 2 discriminant functions in the LDFA on Mn, Ba, Mg and Sr in scales at 6 sites in the Dee catchment (AB, closed circles; AU, open circles; CW, closed triangles; CR, open triangles; LF closed squares and SB, open squares).

Table 7. Cross validation LDFA classification of fish to their site of origin using all elements which showed significant differences among sites (otoliths, n=6; scales n=11; see Table 5) and Ba, Mg, Mn and Sr concentrations only in otoliths (in front of the slash) and scales (behind the slash). *Shows the percentage of individuals correctly classified to their site of origin. Correctly classified individuals are shown in bold. Light, medium and dark grey boxes correspond to pairs of sites in the upper, middle, and lower regions of the catchment respectively.

Site	Elements used in LDFA	No. of fish classified to each site based on element concentrations in otoliths or scales of each fish						n	Correct* (%)
		CW	LF	AB	CR	AU	SB		
CW	All elements	**16/15**	0/0	0/1	0/0	0/0	0/0	16	100/94
	Ba, Mg, Mn and Sr	**15/15**	1/0	0/1	0/0	0/0	0/0	16	94/94
LF	All elements	1/2	**13/12**	0/0	1/0	0/0	0/1	15	87/80
	Ba, Mg, Mn and Sr	1/2	**13/12**	0/0	1/0	0/0	0/1	15	87/80
AB	All elements	0/0	0/0	**9/8**	**2/3**	0/0	0/0	11	82/73
	Ba, Mg, Mn and Sr	0/0	0/0	**7/8**	**4/3**	0/0	0/0	11	64/73
CR	All elements	0/1	1/0	3/0	**9/12**	0/0	0/0	13	69/92
	Ba, Mg, Mn and Sr	0/1	1/0	2/0	**10/12**	0/0	0/0	13	77/92
AU	All elements	0/0	0/0	0/0	0/0	**13/14**	1/0	14	93/100
	Ba, Mg, Mn and Sr	0/0	0/0	0/0	0/0	**13/13**	1/1	14	93/93
SB	All elements	0/0	0/0	0/0	0/0	0/1	**14/13**	14	100/93
	Ba, Mg, Mn and Sr	0/0	0/0	0/0	0/0	0/0	**14/14**	14	100/100

Table 8. Cross Validation and Original (i.e. non-Cross Validation) LDFA and Multinomial Logistic Regression (MLR) classifications of fish to their site of origin based on element concentrations in scales and otoliths. Columns in bold correspond to the LDFA and MLR classification accuracies which are directly comparable.

Site	Otoliths				Scales			
	LDFA cross validated classification for Ba, Mn, Sr, Mg, Pb, Zn (%)	**LDFA original classification Ba, Mn, Sr, Mg (%)**	**Multinomial Logistic Regression Ba, Mn, Sr, Mg (%)**	LDFA Cross Validated classification Ba, Mn, Sr, Mg (%)	LDFA cross validated classification for Ba, Mn, Mg, Sr, Co, Pb, Li, Si, Cu, Zn, U (%)	**LDFA original classification Ba, Mn, Sr, Mg (%)**	**Multinomial Logistic Regression Ba, Mn, Sr, Mg (%)**	LDFA Cross Validated classification Ba, Mn, Sr, Mg (%)
AB	73	**73**	**82**	73	82	**73**	**82**	64
AU	100	**100**	**100**	93	93	**100**	**100**	93
CW	94	**100**	**100**	94	100	**100**	**94**	94
CR	92	**92**	**92**	92	69	**77**	**85**	77
LF	80	**80**	**100**	80	87	**87**	**93**	87
SB	93	**100**	**100**	100	100	**100**	**100**	100
Average	89	**91**	**96**	89	88	**89**	**92**	86

Table 9. Element concentrations in *Salmo trutta* from the Nith catchment (μg/g±1SE). Smolts (n=12) were caught in the lower part of the main channel during their migration to sea. The adults (sea trout) (n=17) were caught at a range of sites in the main channel on return to spawning grounds in the Nith having resided in the marine environment. Elements in *Salmo trutta* scales for which the majority of data are below the LOD are shown in italics. One-way ANOVA results comparing element concentrations in both groups of fish are also shown.

	Element concentrations in *Salmo trutta* scales from the Nith catchment [mean (μg/g)(±1SE), range (μg/g)]				One-way ANOVA	
	Smolt		Sea trout			
Element	Mean	Range	Mean	Range	$F_{1,28}$	P
Mg	4822 (±524)	1760-7884	8495 (±922)	3020-15028	8.03	0.009
Mn	277 (±56)	105-832	199 (±54)	50-1026	0.98	0.332
Co	0.45 (±0.07)	0-0.8	0.7 (±0.20)	0.1-3.7	0.71	0.408
Cu	6.3 (±2.4)	0-31	23 (±11)	0.4-189	1.48	0.235
Zn	880 (±202)	372-2977	613 (±108)	43-2124	1.58	0.219
Sr	556 (±27)	446-779	1066 (±52)	620-1352	69.12	<0.001
Ba	119 (±17)	67-237	52 (±8)	9-138	15.54	0.001
Pb	11 (±4)	1.3-43	15 (±7)	0-134	0.13	0.722
U	*0.45 (±0.23)*	*0-2.4*	*0.33 (±0.2)*	*0-3.1*	0.18	0.671
Li	3.7 (0.6)	0.4-7	2.0 (±1)	0-11	1.67	0.208

Chapter 4

A multi-disciplinary approach to mapping spatial variations in fish scale chemistry in a small river catchment

Abstract

The spatial variability in scale chemistry of *Salmo trutta*, a salmonid fish, in the Dee catchment (drainage area ~1800km^2) in Wales, U.K. was examined. Significant differences were found in concentrations of Ba, Co, Cu, Li, Si, Mg, Mn, Pb, Sr, U and Zn in scales among 12 sites geographically dispersed throughout the catchment. Over 86% of fish could be classified to their site of origin, using cross-validation (CV) Linear Discriminant Function Analysis. Concentrations of Sr and Mn in scales were correlated with concentrations in stream water, providing a unique opportunity to map high resolution catchment-wide variability in scale element concentrations using British Geological Survey (BGS) stream water chemistry data as a proxy for Sr and Mn in scales, at 792 sites in the catchment (neighbouring sites were >41m apart).

We found evidence of regional patterning in Sr and Mn in scales among sites in the upper, middle and lower areas of the catchment (n=12) and 73% of scales could be correctly classified to their region of origin based on these elements alone. However, this regional patterning was not present in predicted scale chemistries at sites in 1st and 2nd order tributaries (n=792). Some geographically isolated sites in the Dee catchment might be expected to show similar concentrations of Sr and Mn in scales. As *Salmo trutta* are known to exploit 1st, 2nd and 3rd order tributaries in the catchment it is unlikely that *Salmo trutta* scale chemistry will be a useful fisheries research tool in the Dee catchment. Using a site-based design to sample structure chemistry at 12 sites did not therefore provide an adequate account of spatial variability in Sr and Mn concentrations in scales in the Dee catchment. Future studies determining baseline element signatures in fish calcified structures should take account of the river network inhabited by the fish species in question (e.g. 1st, 2nd and 3rd order tributaries) and the degree to which structure chemistry might vary at fine spatial scales.

Introduction

The application of 'biogeochemical tags' in ecological research has greatly improved our knowledge of species spatial ecology (Campana, 1999; Hobson, 1999). Biological structures such as teeth, vertebrae, fish scales and otoliths (fish ear bones) incorporate stable isotope ratios and element concentrations derived from the local environment and can be used to reveal migration patterns, habitat fidelity and intermixing of populations (Hobson, 1999; Wells *et al.*, 2003; Barnett-Johnson *et al.*, 2008). Application of the technique for elucidating natal origins and movement patterns of migratory fish has generated much interest (Milton *et al.*, 1997; Wells *et al.*, 2003; Barnett-Johnson *et al.*, 2008). Scales and otoliths (herein described as 'structures') form incremental growth bands throughout the lifetime of fish, which provide a chronological record of element 'impurities' to which the fish has been exposed. The technique has been useful for retrospectively determining the geographical origins of fish stocks (Milton *et al.*, 1997; Barnett-Johnson *et al.*, 2008) and for revealing the periodicity and duration of fish migrations (Campana *et al.*, 1999; Schaffler *et al.*, 2009).

The application of biogeochemical tags depends on prior knowledge of baseline chemical 'signatures' in fish calcified structures from all geographical areas inhabited by fish prior to capture (Campana, 1999). Establishing baseline signatures at each location can be prohibitively time consuming and expensive. As a result, baselines are often estimated by measuring the chemistry of fish structures at a number of sites within the study area (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005). To date, most of the studies on biogeochemical tags of freshwater fish have been conducted in large catchments that drain areas of ~5,000km^2 to >100,000km^2 (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007; Adey, 2007). The baseline signatures are often determined at a low density of sites ($n<40$) in major tributaries in a study area within the catchment. This method rarely accounts for all possible sources of fish and assumes that no inward fish migration occurs from beyond the study boundary. In these cases, the high degree of accuracy with which fish can be classified to their site of origin, is probably dependent on the number of sites included in the study and does not reflect the true accuracy with which the geographical origin of fish can be determined. While these studies have shown that

biogeochemical tags have potential as a freshwater fisheries research tool, they are restricted to evaluating site-specific patterns in fish structure chemistry (Wells *et al.*, 2003; Barnett-Johnson *et al.*, 2008). Occasionally there appears to be some regional patterning in the fish structure chemistry among the sites in the study area (e.g. Bronte *et al.*, 1996; Muhlfeld *et al.*, 2005). However, failure to account for potential fine scale variability in baseline chemical signatures could result in the erroneous classification of fish to site of origin and/or overestimating the accuracy with which spatial movement patterns of fish can be determined (Campana *et al.*, 2000; Gillanders, 2005; Elsdon *et al.*, 2008). Wells and co-authors (2003) suggested that establishing baseline chemical signatures for all stream environments accessible to fish prior to capture is important (Wells *et al.* 2003). More specifically, the study design must determine the geographical areas between which temporally stable differences in baseline chemical signatures exist.

The limitations of a site-based design in establishing baseline signatures have been addressed to a degree in some of the studies that use naturally occurring stable isotope ratios as biogeochemical tags. In a limited number of studies, stable isotope ratios in biological structures have been predicted on the basis of continuous environmental data. For instance, the relationships between ^{87}Sr:^{86}Sr stable isotope ratios in fish otoliths and bedrock geology (Kennedy *et al.*, 2002) and $\delta^{15}N$ & $\delta^{13}C$ in scales and land use (Harrington *et al.*, 1998) have been reported. Recently, one of the first 'isoscape' (i.e. map of predicted stable isotope ratios across a geographical region based on interpolated stable isotope data) studies in an aquatic system successfully predicted the natal origins of Chinook salmon (*Oncorhynchus tshawytscha*) based on the relationship between continuous compositional patterns of bedrock and ^{87}Sr:^{86}Sr in fish otoliths and stream water (Barnett-Johnson *et al.*, 2008).

In contrast to studies using stable isotope ratios as biogeochemical tags, there has been little attempt to predict the variability in element *concentrations* in fish structures based on environmental data, despite the wealth of literature on element concentrations as biogeochemical tags. This may reflect a lack of appropriate data and/or the complexity of factors which are known to influence the rate at which elements are incorporated into fish structures including factors such as temperature, salinity and physiological differences among fish (Fowler *et al.*, 1995; Elsdon & Gillanders, 2004; Elsdon & Gillanders, 2005) (Appendix B). However, the relationship between element concentrations in fish structures and water has been

demonstrated under both laboratory (Wells *et al.*, 2000; Elsdon & Gillanders, 2004; Elsdon & Gillanders, 2005), and field-based conditions (Wells *et al.*, 2003; Elsdon & Gillanders, 2005). The most consistently reported of these relationships is for Sr (e.g. Wells *et al.*, 2000; Elsdon & Gillanders, 2005; Muhlfeld *et al.*, 2005), which is often found to be a key element in discriminating fish origins (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005).

This study evaluates the role that stream water chemistry data collected at a fine spatial scale might have in mapping spatial variability in fish-structure chemistry. The findings will be compared with those achieved using the traditional site-based approach for determining baseline chemical signatures. The study will be carried out in the Dee, a small catchment in Wales, U.K. (Fig. 1) and will sample fish scale chemistry in the major 3rd order tributaries. Fine-scale variability in structure chemistry throughout the catchment will then be predicted by using stream water chemistry data collected from 792 sites in 1st, 2nd and 3rd order tributaries (BGS, 1999), as a proxy for fish scale chemistry. Stream water chemistry has already been used as a proxy for scale chemistry in order to improve the spatial coverage of sites by Wells *et al.* (2003), but the density of sites in this study was still low (n=<40), in relation to the size of the study area (~5000km^2). Given that the Dee catchment drainage area is ~1800km^2, the existing stream water data from 792 sites provides a unique opportunity to map catchment-wide variability in scale chemistry at a fine spatial resolution (mean distance between neighbouring water sampling sites: ~738m, range: 41-3634m). Potentially, this approach could provide near-continuous baseline chemical signatures for the entire Dee river system. The stream water data was collected as part of the Geochemical Baseline Survey of the Environment (GBASE) carried out between 1988-1994 by the British Geological Survey (BGS) (BGS, 1999). BGS research has shown that bedrock geology is the principal driver of regional variation in concentrations of certain elements in stream water in Wales (e.g. Sr and Ba) (BGS, 1999). Given that bedrock is temporally stable, the BGS stream water data should be representative of concentrations of these elements in the Dee catchment today.

Salmo trutta is an excellent candidate species for studies on biogeochemical tags, due the continuum of life history strategies that the species exhibits (Cucherousset *et al.*, 2005), which can involve migration/movement over considerable distances (Ovidio *et al.*, 1998). The species is phenotypically plastic

and can reside in freshwater during its entire life or utilise a combination of freshwater, estuarine and marine habitats (Jonsson, 1989; Cucherousset *et al.*, 2005). This study examines the application of biogeochemical tags to elucidate the natal origins and movement patterns of the species during its freshwater life stages. At present relatively little is known about how *Salmo trutta* utilise the freshwater river network available to them. The interconnected nature of river channels mean that thousands of kilometres of river could be potentially accessible to fish in a given catchment. While it has been established that *Salmo trutta* revisit their natal tributaries to spawn and may travel >20km over several weeks to do so (Ovidio *et al.*, 1998; Rustadbakken *et al.*, 2004), we do not know the distance or periodicity of movements of freshwater *Salmo trutta* in their search for feeding and refuge areas, or the degree to which reproductively isolated populations interact and how these behaviours change throughout the lifetime of fish. Understanding the extent to which fish exploit river networks is key to determining the vulnerability of populations and is critical for ensuring connectivity between *Salmo trutta* habitats. Biogeochemical tags could provide a useful tool with which to reveal natal origins and movements of fish, providing that temporally stable, geographical boundaries exist between different baseline chemical signatures in fish structures. The objectives of the study are to evaluate spatial variability in fish-structure chemistry using a traditional site-based design for sampling fish-structure chemistry and compare this with the results achieved when element concentrations are predicted at 792 sites in a catchment.

Methods

The research was carried out in the Dee catchment in Wales, an important *Salmo trutta* habitat in the U.K., that has freshwater-resident *Salmo trutta* in addition to an annual estimate of *ca.* 9,300 sea-run *Salmo trutta* (for the period 1992-2003) (Davidson *et al.*, 2007). The catchment drains an area of ~1800km^2. The headwaters lie in the upland Cambrian mountain range, and the main river drains through the lowland Cheshire basin before discharging into the Irish Sea at Liverpool Bay. Ordovician and Silurian bedrock dominate in the SW, and Carboniferous and Triassic bedrock dominate in the NE of the catchment (Fig. 1). In a previous study it was established that recently deposited hydroxyaptite in scales of *Salmo trutta* in the

Dee catchment offered a non-lethal alternative to the examination of aragonite in otoliths as a record of spatial variations in some element concentrations in fish-structures (Chapter 3). To examine variability in scale chemistry in the catchment using a site-based design, *Salmo trutta* scale samples were collected from a geographical spread of sites in 3rd order tributaries for chemical analysis. To confirm that stream water chemistry could be used as a proxy for scale chemistry, the element concentrations in stream water at each site were also analysed (see below).

Fish collection was carried out at 19 sites in the Dee catchment between August and September 2007 (Fig. 1), using an InteliSYS Fish Magnet Mobile 11 (FMM) electrofisher back-pack. Fish were anesthetised using an approved U.K. Home Office Schedule 1 method. *Salmo trutta* were encountered at 12 of the 19 sites (Fig. 1) and scale samples were removed from 261 fish in total, with sample sizes at each site ranging between 15 – 29 individuals (Table 1). Onsite, fish fork length was measured and scale samples were removed from above the lateral line, slightly posterior to the dorsal fin, on the left side of each fish and stored in manila envelopes. All equipment in contact with water and scale samples was new for this experiment, non-metallic, acid-washed in 10% nitric acid (TraceMetal Grade, Seastar Chemicals Inc.), triple-rinsed in 18.2MΩ water (Milli-Q), dried in a laminar low hood and stored in sealed plastic bags prior to use.

Scales were prepared for analysis using similar methods to those outlined by Wells *et al.* (2003). One non-regenerated, undamaged scale from each fish was manually cleaned under a dissecting microscope using nylon brushes and plastic-tipped forceps. These implements were acid-rinsed in 10% nitric acid (Trace Grade, Seastar Chemicals Inc.) between uses. Samples were ultrasonically cleaned for 4min in trace element grade 3% hydrogen peroxide (Trace Grade, Fluka), triple-rinsed with 18.2MΩ water (Milli-Q) and dried overnight in a laminar flow hood. Samples were randomised to create blocks of 12 samples with each block containing one randomly chosen sample from each site. One scale per fish was mounted onto glass slides, using double sided tape (No More Nails Tape), so that the hydroxyapatite layer was exposed for LA-ICP-MS analysis. The tape had previously been analysed by LA-ICP-MS to confirm that elements of interest were low or below detectable levels (see Appendix E). Three replicate spot ablations, each 122μm in diameter, were carried out on the distal (outer) edge of each scale (standardised to the same growth axis among scales), corresponding to the most recent (2007) summer growth,

representing scale material most likely to have been formed at or close to the site of capture (Elsdon & Gillanders, 2005; Hamer & Jenkins, 2007).

Scales were analysed with a 193nm ArF Geolas Q Plus exciplex laser ablation facility coupled to an Agilent 7500c Quadrupole Inductively Coupled Plasma Mass Spectrometer (LA-ICP-MS). During scale analysis, the laser was set to a 100 pulse count and fired at a rate of 5Hz. The energy of the laser was 6 J/cm^2. The focus of the laser could be controlled to an accuracy of ~1μm which allowed the scale hydroxyapatite layer to be removed from the basal plate. The ICP-MS was set to an integration time of 10ms. Blanks were measured before every 3rd sample by running the ICP-MS for 20s prior to laser ablation. NIST Standard Reference Material (SRM) 610 was used to calibrate the ICP-MS facility and NIST 610 and 612 were analysed as 'unknowns' three times during each day of analysis. Examination of the NIST 610 and 612 data revealed no systematic instrument drift. In addition to ^{44}Ca, the following isotopes were included in the analysis: ^{7}Li, ^{24}Mg, ^{28}Si, ^{55}Mn, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{138}Ba, ^{208}Pb, and ^{238}U.

Triplicate 100ml samples of stream water were collected at each site on 5th and 6th of September 2007, to coincide with base flow conditions (Muhlfeld *et al.*, 2005; Veinott & Porter, 2005) for that summer. Water samples were syringed through a 0.45 μm membrane, into polypropylene bottles, acidified with 0.5ml of 70% ultrapure nitric acid (Optima, Seastar Chemicals Inc.) and refrigerated at 4°C until analysis. All equipment which came into contact with the stream water was prepared in the same manner as equipment used during the scale sample preparation (described above). Powder-free vinyl gloves were worn during water sampling. Water samples were analysed using a VG Elemental PlasmaQuad II+ ICP-MS. Three external multi-element standards and an internal standard of Ru were used in the analysis. Duplicate analyses were carried out on each sample. Duplicate analyses were averaged and then triplicate samples were averaged to get a single element concentration in stream water for each site. The GBASE streamwater chemistry data was provided by the BGS [see Simpson *et al.* (1993) and BGS (1999) for details on the analytical methods used in the GBASE study].

Data Processing

Scale LA-ICP-MS data were processed using SILLS Project software (Mineralogical Association of Canada Short Course 40, Vancouver, B.C.). A plot of

counts per second for each isotope recorded during each ICP-MS run was used to isolate element concentrations corresponding to scale hydroxyapatite removed during each ablation. Raw data were blank corrected. The Limit of Detection (LOD) was calculated as the blank value plus three standard deviations (SDs) of the blank (Miller & Miller, 1993). Ca was used as an internal standard as it is a major component of the hydroxyapatite layer in scales and typically its concentration varies by <1% (Clarke *et al.*, 2007). Concentrations of the remaining elements in the analysis were estimated in relation to concentrations of Ca and reported in μg/g of hydroxyapatite (Flem *et al.*, 2005). Hydroxyapatite is not a stoichiometric crystal and so the value of 374,000μg/g (±4,000μg/g) was used for the Ca content of salmonid scale hydroxyapatite as reported in a previous study (Flem *et al.*, 2005). The data for each set of three replicate ablations were averaged to provide one element concentration for each scale. Outliers were identified as values greater than five standard deviations away from the mean for that element (Veinott & Porter, 2005). Less than 2% of the data for any element were classed as outliers and these were replaced with the mean value of that element, pooled across all sites.

Analytical precision during the scale analysis was calculated as the relative standard deviation (RSD) of element:Ca in the NIST SRMs and was <10% for all elements except for Zn which exhibited relatively poor precision for NIST 610 (17% RSD) and NIST 612 (20% RSD). However, Kruskal-Wallis revealed that significant differences in Zn in scales were still detectable among sites ($H=52.55$; $df = 11$; $P<0.001$) and this element was therefore kept in the analysis. Recovery of elements for the NIST SRMs, based on published element concentrations (Pearce *et al.*, 1997), ranged between 99-110% for all elements except for Mg (85%) and Zn (115%) in the NIST 612 (N.B. no published element concentrations for Si in the NIST SRMs were available). Where ablation data for an element fell below the LOD, an element concentration for a given sample was derived from the remaining replicate ablations, providing that the element's concentration was above the LOD for at least one ablation. The percentages of scale samples for which all three replicate ablations fell below the LOD for a given element were: Li 13.5% and U 2.3%. Occasionally, data that fell below the LOD were negative after blank correction in which case a positive constant, corresponding to the largest negative value plus 1 was added to the data to allow $\log_{10}$ transformation (Fowler *et al.*, 1995).

Log_{10} element concentrations were tested for normality using Anderson-Darling's (AD) test. All log_{10} element concentration data failed normality tests. However, graphical inspection of the element data revealed relatively minor deviations from normality (McGuinness, 2002). The log_{10} element concentration data were also tested for homoscedasity using Levene's test. Zn and Pb data failed homoscedasity tests.

Element concentrations in calcified structures have been shown to vary with fish length/otolith size (Thorrold *et al.*, 1998; Brophy *et al.*, 2003). In the present study, fish fork length was used to examine the effect of fish size/age on element concentrations. ANCOVA was used to reveal common within-group regression slopes of element concentration and fork length at each site by using each element in the scales as the response variable, site as a category variable and fork length as a covariate. Providing that the assumption of equal slopes across sites was met, the effect of fork length on element concentrations could be removed by using the common within-group regression coefficient in the following equation, adapted here from Almeida *et al.* (2008):

$$AC_{ij} = UAC_{ij} - [\beta \times (Log_{10}FL_j - Log_{10}\underline{FL})]$$

where AC_{ij} is the adjusted transformed element concentration for element i of the j specimen, UAC_{ij} is the unadjusted character measurement for element i of the j specimen (UAC was log_{10} transformed), β is the equal common within-group regression coefficient of element i (log_{10} transformed) regressed against fork length (log_{10} transformed), FL_j is the fork length of the j specimen (log_{10} transformed) and $\underline{FL}$ is the mean fork length of all fish (log_{10} transformed).

The ANCOVA was repeated (this time with the adjusted element concentration as the response variable) to confirm that the regression slope between fork length and element concentration was no longer significant ($P>0.05$). Within-site regressions of adjusted element concentration were used to confirm that length was no longer a significant covariate. The adjusted element concentration values were used in all subsequent analysis. ANCOVA is robust to departures from normality providing data are homoscedastic (Olejnik & Algina, 1984) and it has been used to correct for size effects on non-normal data (Claytor *et al.*, 1991; Almeida *et al.*, 2008). Log_{10}Zn data were both non-normal and heteroscedastic.

However, ANCOVA has been demonstrated to remain robust provided that sample sizes are large (Olejnik & Algina, 1984)

One-way ANOVA was used to examine whether there were significant differences in element concentrations in scales among sites. Non-normality does not preclude the use of ANOVA, providing data are homoscedastic (McGuinness, 2002). Post-hoc tests using Scheffe's test, which is relatively insensitive to non-normally distributed data, was used to examine which pairs of sites differed significantly in element concentrations in scales. Kruskal-Wallis one-way analysis of variance was used for $\log_{10}$Zn and $\log_{10}$Pb data as these elements were both non-normally distributed and heteroscedastic. Tamhane's post-hoc test was used for these elements as it is insensitive to heteroscedastic data.

Linear Discriminant Function Analysis (LDFA) was used to evaluate the accuracy with which fish could be classified to their site of origin, based on element concentrations in scales. Discriminant Function Analysis is routinely used in stock discrimination studies (discussed in: Prager & Fabrizio, 1990; White & Ruttenberg, 2007), and is commonly used in studies identifying stocks of fish based on scale or otolith chemistry (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007). Minor deviations from normality do not preclude the use of LDFA (Leakey *et al.* 2008). The LDFA involved an 'original' classification (which used the discriminant functions to classify the same samples that were used to develop the functions) and a 'cross-validation' (CV) classification (which involves leaving one sample out of the dataset before establishing the discriminant functions and then classifying the sample that was removed). Collinearity was detected among elements and therefore a stepwise LDFA was avoided and non-stepwise LDFA Standardised Discriminant Function Coefficients used to indicate the relative importance of element concentrations in the LDFA classification (Sharma, 1996). Cohen's Kappa statistic was used compute the chance-corrected agreement between actual and predicted group (site) memberships of fish (Titus *et al.*, 1984; Barnett-Johnson *et al.*, 2008). The Kappa statistic ranges between 0 (indicating the classification to site was no improvement over that achieved by chance) and 1 (indicating that there was perfect agreement in the classification to site when taking into account classification by chance). Given that some of the element concentration data failed assumptions of LDFA, a non-parametric Multinomial Logistic Regression (MLR) was used to verify the classification accuracy achieved with the LDFA.

To determine whether stream water chemistry could be used as a proxy for scale chemistry, Pearson's Product Moment correlation was used to examine the relationship between mean element concentrations in scales at each site (Walther & Thorrold, 2008) and element concentration in stream water data collected in 2007 and in 1988-1994 (BGS, 1999). The 1988-1994 sample sites in each site sub-catchment (i.e. the geographical area draining to each site) (Fig. 2) were averaged to provide a single value for each site for correlation analysis. The correlations were carried out with the stream water data expressed as both absolute concentrations (μg/L) and as element:Ca ratios. No BGS data were available for the EA site sub-catchment (Fig. 2) and so correlations were based on stream water element concentrations at 11 sites only.

To determine if element concentrations in streamwater were temporally stable in the Dee catchment, stream water data collected in 2007 and 1988-1994 were compared by correlation analyses.

Regression equations for element concentrations in scales against element:Ca in streamwater collected in 2007 were used to predict element concentrations in scales at 792 sites in the Dee catchment, using the BGS stream water data as a proxy for scale chemistry. Regressions were carried out on element:Ca in stream water at each site and element concentrations in individual scales (rather than using mean element concentrations in scales at each site) in order that the regression equations took into account the variability in element concentrations in scales at each site. The regression equations were used to predict scale chemistry at the BGS survey sites (n=792, mean distance between neighbouring sites = 738m, range = 41-3634m) for those elements in *Salmo trutta* scales which were correlated with water chemistry in both the 2007 and 1988-1994 streamwater datasets. ArcMAP was used to display spatial variability in predicted baseline chemical signatures in scales. Predicted element concentrations in scales at the 792 sites were also plotted against longitude in order to determine if there was any regional patterning in scale chemistry.

The relationship between element concentrations in stream water and existing environment data such as percentage area of land use (CCW, 2008), bedrock (BGS, 2008)(Fig. 1) and superficial geology (BGS, 2008) in the site catchments were analysed using multiple regression. This allowed the temporal stability of water/structure chemistry to be assessed by establishing whether bedrock heterogeneity explained a high proportion of the variability in element

concentrations in stream water in the catchment. Topographical contour data (OS, 2009) and river network data (CEH, 2007) were then used to identify the watershed of each survey site. These boundaries were used to isolate the area of landuse/bedrock geology/superficial geology types within each site sub-catchment (Fig. 1). Bedrock types were grouped according to geochronological time period (Carboniferous, Ordovician, Silurian, Triassic and 'other') as these groups have been found to influence element concentrations in stream water in the study region (BGS, 1999).

All statistical analysis was carried out in SPSS (v. 16) and Minitab (v.14).

Results

Site locations and fork length of *Salmo trutta* collected in the Dee catchment in 2007 are shown in Figure 1 and Table 1 respectively. Significant differences in fish fork length were found among the 12 sites ($F_{11,260}$ = 10.87; P <0.001). Statistically significant common within-group regression slopes were found for fish fork length and $\log_{10}$Zn (β= -0.40; P=0.012) and $\log_{10}$Si (β= -0.62; P<0.001) in scales. The effect of fork length on these elements was removed (see methods section) and the adjusted data used in subsequent statistical analysis.

Classifying fish to site of origin based on element concentrations in scales.

Mean element concentrations in scales at each survey site are shown in Table 2. There were significant differences in all $\log_{10}$ element concentrations among sites (Table 2). Sr was significantly different among the highest number of pairs of sites (48 out of 66 pairs) and all but one of those pairs were highly significantly different (P<0.001). Ba and Mn were significantly different between 33 and 32 pairs of sites respectively (Table 2). The findings suggest that these three elements might be important in discriminating fish origin in the Dee catchment, based on scale chemistry. 86% of fish could be classified to their site of origin using cross-validation (CV) LDFA. Classification accuracy at the site level ranged between 67% (Site: ML) to 97% (Site: AU)(Table 3). Cohen's Kappa statistic was 0.87 (±0.4 CIs). The standardised canonical discriminant functions indicate that Sr and Mg were the most important elements in the first discriminant function which explained 59% of

the total variability in scale chemistry (Table 4). The MLR classified 97% of fish to their site of origin.

Relationship between scale and steam water chemistry.

Concentrations of Sr in scales were positively correlated with Sr:Ca in 2007 stream water ($r = 0.69$; $df = 11$; $P = 0.012$) and the BGS stream water ($r = 0.59$; $df = 10$; $P = 0.055$) and concentrations of Mn in scales were correlated with Mn:Ca in both the 2007 ($r = 0.851$; $df = 11$; $P < 0.001$) and the BGS streamwater ($r = 0.787$; $df = 10$; $P = 0.004$), despite differences in the timing and location of water sample collections. No other elements that were analysed in scales were correlated with both the 2007 and BGS stream water datasets.

Temporal stability of Ca, Sr and Mn in stream water

Absolute concentrations of Ca and Sr in water were correlated between the 2007 and BGS datasets and exhibited relatively minor deviations from a 1:1 relationship (Fig. 3). No relationship was present for Mn ($r = 0.24$; $df = 10$; $P = 0.48$). However, there was a significant positive correlation for Mn:Ca in 2007 and BGS stream water (Fig. 3) although this relationship deviated from a 1:1 relationship.

Figure 4 shows that both Ca and Sr concentrations follow a very similar spatial pattern in concentrations in the 2007 and 1988-1994 datasets, despite the difference in the timing and location of the sample collections. Ca and Sr concentrations are higher in the NE area of the catchment where the bedrock geology is predominantly Carboniferous and Triassic (Fig. 4) (BGS, 1999). Mn appears less clearly related to bedrock geology (Fig. 4). However, when the relationship between element concentrations in 2007 stream water samples and bedrock geology was examined using multiple regressions, the composition of bedrock within each site sub-catchment (Table 5) explained a large percentage of the variability in element concentrations in stream water of Mn (R^2 adj = 72%, $F_{3,11}=8.15$, $P=0.009$) in addition to Ca (R^2adj = 96%, $F_{3,11}= 60.31$, $P<0.001$) and Sr (R^2adj=88%, $F_{3,11}= 21.30$, $P=0.001$). N.B. Ordovician was excluded from the multiple regressions because it was negatively correlated with the percentage cover of Carboniferous bedrock (Spearman's rank $r=-0.69$; $df=11$; $P=0.014$).

Ca, Sr, and Mn:Ca in stream water appear to be relatively temporally stable, suggesting that the 1988-1994 stream water dataset can be used as a proxy for scale chemistry.

Fine spatial variability in Sr and Mn in scales in the Dee catchment.

A plot of Sr and Mn concentrations in scales indicates that fish from sites in the upper, middle and lower regions of the Dee catchment might be distinguishable, based on concentrations of these elements in scales (Fig. 5). Figure 6 shows that there are high concentrations of Mn:Ca in the upper region (West) of the Dee catchment and lower concentrations in the middle and lower regions (except for site EA). While Sr shows less regional patterning in scales, concentrations tend to be higher in the upper and middle region of the catchment. 73% of fish could be correctly classified to their region of origin using CV LDFA based on Sr and Mn in scales pooled across sites in each region (upper region sites; LF, MN, ML, CW, CD, middle region sites; MW, AB, Cr and lower region sites; AU, AD, EA, SB). The Cohen's Kappa statistic when using all elements in the LDFA was 0.83 (±4 CIs).

To explore whether this regional patterning was present at a fine spatial resolution in the catchment, Sr:Ca and Mn:Ca in BGS stream water data were used to predict variability in scale chemistry at 792 sites in the Dee catchment (Fig. 2). The following regression equations were used to convert the BGS stream water data to predicted element concentrations in scales:

$$Mn_{scale}\ (\log_{10}) = (0.345 \pm 0.02SE) \times \log_{10}Mn{:}Ca_{water} + (3.27 \pm 0.07SE)$$

$$(r^2 = 0.46, P < 0.001)$$

$$Sr_{scale}\ (\mu g/g) = (8953 \pm 627SE) \times Sr{:}Ca_{water} + (217 \pm 6.8SE)$$

$$(r^2 = 0.44, P < 0.001)$$

The converted BGS data were displayed in ArcMAP to visualise the fine-scale spatial variability in predicted scale chemistry throughout the Dee catchment (Fig. 7). Figure 7 shows that some geographically isolated sites probably share similar element concentrations in scales. While Mn concentrations are generally higher in the West, the scatter plots in Figure 8 indicate a wide range of Mn concentrations would be expected in scales in the upper region of the catchment region. Figures 7 and 8 indicate little evidence of regional patterning in Sr concentrations within the Dee catchment. The regional patterning found in Sr and Mn concentrations in scales at the 12 sites in 3rd order tributaries is therefore not reflected in high resolution spatial variability in the concentrations of these elements in 1st and 2nd order tributaries.

Discussion

The majority of studies using fish structure chemistry as biogeochemical tags for researching natal origins of migratory fish and movements patterns in freshwater, have been carried out in large catchments using samples collected from a low density of sites (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005). The BGS water chemistry data provided a unique opportunity to predict spatial variability in concentrations of Sr and Mn (two principal elements for discriminating fish between the 12 sites in this study), at a high density of sites (n=792) in the Dee catchment.

The scale-sampling study design was similar to that used in other studies, where fish calcified structures (in this case scales) were collected from a small number of geographically spread sites in major tributaries (in this case in 3rd order tributaries) within the catchment. The initial results from the scale chemistry analysis were encouraging. Significant differences in element concentrations were found for a suite of elements among sites, some of which have been important discriminators in other studies, e.g. Ba, Sr and Mn (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005). In addition, differences were found among sites for other, less well documented elements, such as U and Co. The classification accuracy was comparable to that achieved in other studies which have used a site-based design (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005). These initial results are particularly encouraging, given that the study was carried out in a small catchment (Wells *et al.*, 2003; Muhlfeld *et al.*,

2005) and demonstrated that detectable differences in element concentrations occur over very small spatial scales in an upland catchment, supporting earlier pilot work carried out in the Dee (Chapter 3).

However, the spatial distribution of the 12 sites in the Dee catchment, did not account for all sources of fish within the system. If natal origin is to be determined for *Salmo trutta*, which can spawn in very small tributaries (Bembo *et al.*, 1993), it is necessary that the chemistry of scales show some degree of regional patterning within the catchment. Sr and particularly Mn concentrations in scales did indeed show some indication of regional patterning. High Mn:Ca in stream water and scales from sites in the upper regions of the catchment are probably due to relatively low concentrations of Ca in this region (sites LF, MN, ML, CW and CD)(Fig. 4).

When stream water was used to predict scale chemistry at 792 sites across the catchment, relatively high concentrations of Mn in scales were predicted to occur in the upper region of the catchment. However, the wide range of concentrations of predicted Mn in scales in this region (Fig. 7 and 8) suggests that there are areas of the upper catchment which, despite being geographically isolated from the middle and lower regions, would be expected to share similar concentrations of Sr and Mn in scales. This suggests that it could be difficult to distinguish fish from these areas, based on scale chemistry alone.

It was beyond the scope of the current study to undertake an evaluation of intra-tributary variability in scale chemistry, but scale chemistry at two sites on the Alyn tributary (sites AU and AD) had significant differences in Sr, Ba and Pb concentrations in scales between sites, indicating that complex intra-channel variability in scale chemistry may exist in the catchment, and this would be expected to further complicate the process of determining baseline signatures in scales.

It is important that baseline chemical signatures remain stable on a timescale exceeding that of the study or even the lifespan of the target species if historical archives of otoliths/scales are to be used. Studies examining temporal stability in element concentrations in fish structures in freshwater are limited in number and tend to be carried out over relatively short timescales (e.g.1-2 years (Wells *et al.*, 2003). Significant differences in otolith/scale chemistry have been found among years (Muhlfeld *et al.*, 2005). In this study, the strong positive correlations between Ca, Sr and Mn:Ca in the 2007 stream water dataset and the BGS stream water dataset indicate that these elements might be sufficiently temporally stable in the Dee

catchment to maintain general spatial trends in the concentration of these elements in the catchment over decadal timescales. While the relationship between Ca and Sr in the two datasets approximates a 1:1 relationship, this was not the case of Mn:Ca which showed higher ratios in the BGS dataset when compared with the 2007 stream water dataset. This is probably because the BGS dataset comprises stream water samples collected predominantly from 1st order tributaries in each site sub-catchment. Mn tends to rapidly decrease in concentration downstream due to hydrolysis (Abdullah & Royle, 1972) and precipitation. This is known to occur in the Dee catchment (Abdullah & Royle, 1972) and may explain why Mn:Ca are higher in the BGS dataset compared with the 3rd order tributaries sampled in the 2007 dataset. The 2007 and the BGS water samples were collected during base flow conditions and therefore should be representative of average element:Ca ratios during the optimal season of growth (Muhlfeld *et al.*, 2005).

The relationship between bedrock and element concentrations in stream water in 2007 and the BGS dataset, for Mn and particularly Sr and Ca is a further indication that element concentrations might be temporally stable in the Dee. The relationship between bedrock and stream water chemistry in the region is well documented (BGS, 1999) and our 2007 stream water chemistry results corroborate with other studies carried out in the region, which show that Ca concentrations are lowest in the Ca-poor igneous and sedimentary rocks in the SW, which range from Ordovician to Silurian in age (Simpson *et al.*, 1993; BGS, 1999). Sr showed a similar distribution to Ca in the catchment, while Mn was less dependent on geology (BGS, 1999).

A number of studies have been carried out on temporal variability in stream water chemistry in North Wales. Ca can show seasonal patterns of high summer and low winter concentrations (Simpson *et al.*, 1993), in contrast Mn, tends to show the opposite pattern (Simpson *et al.*, 1993; Neal *et al.*, 1997), corresponding to seasonal changes in river discharge. While there is evidence of variability in element concentrations in stream water among seasons and years, spatial patterns in stream water chemistry in the region are thought to be robust to these temporal changes (Simpson *et al.*, 1993; BGS, 1999). The range of seasonal variation in element concentrations has not been quantified in this study, but caution should be taken in extrapolating the results between seasons. Consideration should also be given to whether the chemical signature, once deposited in an individual's scale, changes

with time (Campana & Neilson, 1985). However, a previous study has demonstrated that Mn and Sr in scales and otoliths of the same fish were highly correlated (Chapter 3) and so the findings presented here should be applicable to otoliths, which are considered metabolically inert and therefore unlikely to change in chemical composition once formed (Campana & Neilson, 1985).

This is the first study to use scale chemistry data or stream water chemistry data to predict variability in element concentrations in calcified structures of fish, at a fine spatial resolution. The majority of studies examining scale chemistry have sampled scales and/or stream water at a low density of sites, relative to the size of the catchment. This study found some evidence of regional patterning in scale chemistry based on samples from 12 sites in some of the larger tributaries (3rd order), but this patterning was not present in stream water chemistry in 1st and 2nd order tributaries. Given that *Salmo trutta* utilise 1st, 2nd and 3rd order tributaries in the Dee catchment (Environment Agency, 2007), scale chemistry is unlikely to offer a useful biogeochemical tag of *Salmo trutta* in the Dee catchment because the species has the potential to inhabit sites that are geographically isolated but which share similar scale chemistries (i.e. in 1st order tributaries). However, fish structure might provide a useful biogeochemical tag of other anadromous fish species [e.g. Atlantic salmon (*Salmo salar*)] which might inhabit primarily 3rd order tributaries, as these tributaries appear to show some degree of regional patterning. The present study indicates that sampling design needs to take account of the river network inhabited by the fish species in question and the degree to which structure chemistry might vary at different spatial scales. A hierarchical sampling design examining samples collected at a range of spatial scales (at the drainage, tributary and intra-tributary level) (Muhlfeld *et al.*, 2005) and temporal scales (among seasons, months and weeks) (Elsdon & Gillanders, 2006) might offer the best way of identifying whether temporally stable regional differences in calcified structure chemistry exist.

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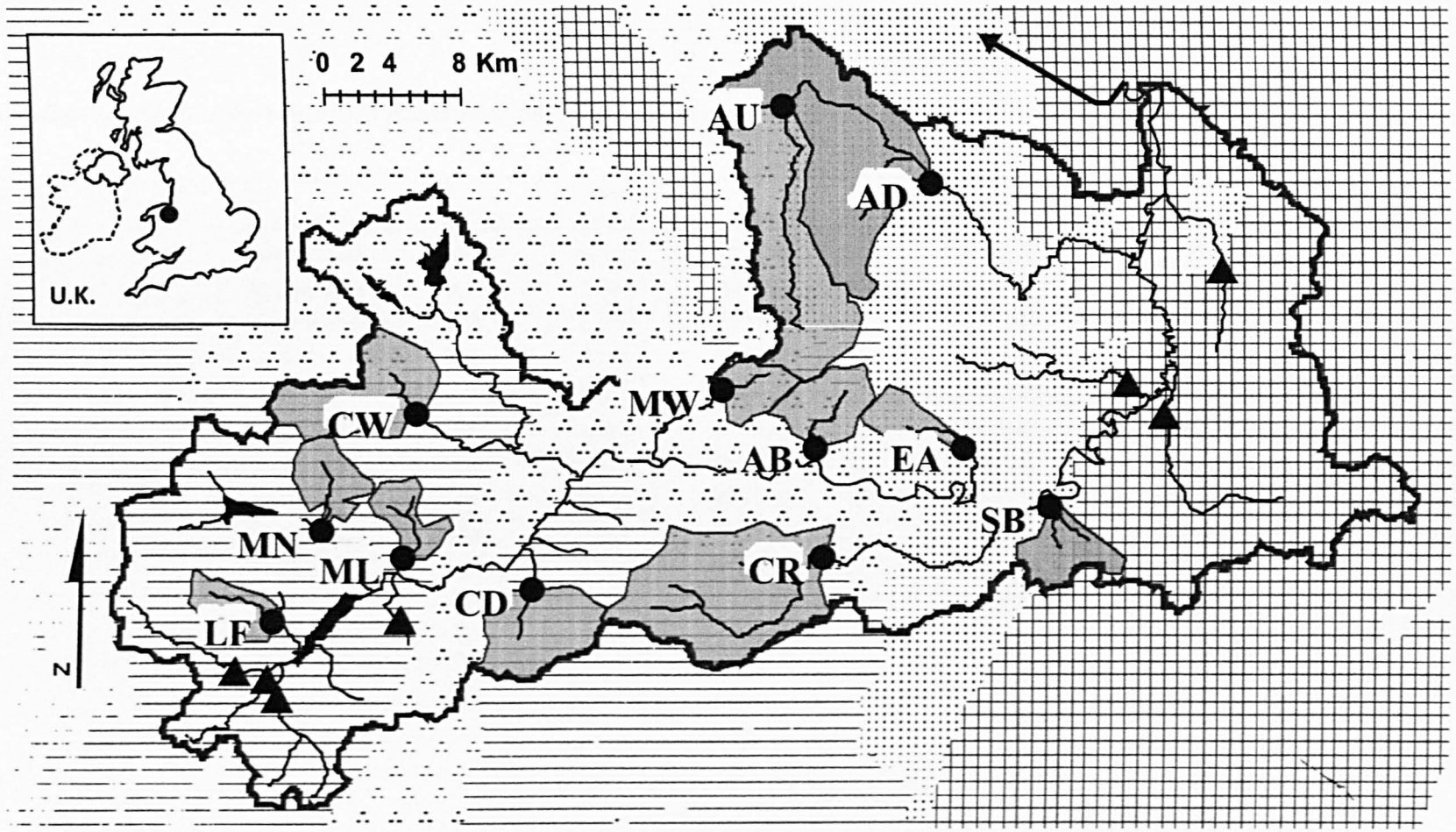


Figure 1. Map of Dee catchment and main river network. The 2007 survey sites (with site sub-catchment boundaries shown in grey) from which *Salmo trutta* scales were collected (black circles), are shown (site names are shown in Table 1). Sites where insufficient numbers of fish were encountered (black triangles) are also shown. Bedrock geology in the catchment (© Crown Copyright/database right 2008. An British Geological Survey/EDINA supplied service) comprises predominantly of Ordovician (horizontal lines), and Silurian (clustered dots in groups of three) in the South West abutting Carboniferous (regularly spaced dots) and Triassic bedrock (cross hatch) to the East (other bedrock types are shown in white).

Table 1. Site name, code, location [British National Grid Reference (NGR) and region within the Dee catchment], sample size (n) and fork length (FL) of *Salmo trutta* collected from 12 sites in the Dee river catchment.

Site Name	Site Code	Catchment Region	NGR	n	Mean FL (±SE) (mm)	FL Range (mm)
Abbey Brook	AB	Middle	SJ206444	23	133 (±5)	76-181
Alyn Upstream	AU	Lower	SJ188653	29	197 (±8)	132-262
Alyn Downstream	AD	Lower	SJ277605	20	227 (±8)	158-302
Ceirw	CW	Upper	SH962465	22	152 (±9)	82-250
Ceidiog	CD	Upper	SJ032357	22	152 (±9)	100-238
Ceiriog	CR	Middle	SJ211377	22	153 (±8)	107-295
Eitha	EA	Lower	SJ296444	21	160 (±6)	126-217
Llafer	LF	Upper	SH877338	15	148 (±10)	86-257
Mynach	MN	Upper	SH906393	21	164 (±9)	122-278
Meloch	ML	Upper	SH955377	21	154 (±7)	123-250
Morwynion	MW	Middle	SJ149478	21	140 (±6)	104-218
Shellbrook	SB	Lower	SJ348407	24	167 (±6)	109-288

Table 2. Mean element concentrations in scales (μg/g±1SE) of *Salmo trutta* and stream water (μg/L) at 12 sites in the Dee catchment. Results for the ANOVA/Kruskal-Wallis test for $\log_{10}$ element concentrations in scales from each of the 12 sites are presented. The number of significant Scheffe/Tamhane post-hoc comparison results between pairs of sites (n=66) is shown. Site names are shown in Table 1. Italic text denotes element concentrations that are below the Limit of Detection (LOD).

Site		Element concentrations in scales (μg/g) (±1SE) and streamwater (μg/L)											
		Li	Mg	Si	Ca	Mn	Co	Cu	Zn	Sr	Ba	Pb	U
AB	Scales	3.0 (±0.3)	4122 (±542)	3201 (±196)	374000	136 (±16)	0.79 (±0.13)	4 (±0.5)	766 (±85)	307 (±18)	26 (±1)	8 (±2)	0.05 (±0.008)
	Water	No data	3403	No data	27450	15	*0.187*	1.7	*2.1*	123	19	*0.19*	0.20
AD	Scales	0.49 (±0.1)	5070 (±362)	2747 (±221)	374000	115 (±8)	0.58 (±0.08)	6 (±2.4)	492 (±52)	224 (±5)	29 (±2)	30 (±5)	0.06 (±0.006)
	Water	No data	4984	No data	58620	12	*0.483*	4.5	3.5	212	60	1.30	0.84
AU	Scales	0.5 (±0.1)	5292 (±326)	3270 (±216)	374000	82 (±6)	0.97 (±0.09)	4 (±0.4)	668 (±63)	187 (±4)	49 (±3)	155 (±33)	0.07 (±0.009)
	Water	No data	2531	No data	18530	5	*0.622*	3.9	*2.3*	53	61	0.44	0.17
CD	Scales	2.87 (±0.3)	4408 (±412)	2811 (±204)	374000	288 (±26)	0.58 (±0.08)	5 (±0.6)	1024 (±176)	420 (±9)	107 (±8)	6 (±2)	0.02 (±0.002)
	Water	No data	1297	No data	999	4	*0.026*	0.9	1.8	16	20	*0.19*	*0.00*
CR	Scales	1.4 (±0.2)	3866 (±416)	3476 (±360)	374000	173 (±17)	0.76 (±0.08)	7 (±2)	1059 (±128)	383 (±7)	29 (±3)	6 (±2)	0.03 (±0.004)
	Water	No data	2001	No data	2247	3	*0.108*	1.7	*1.9*	42	10	0.47	*0.01*
CW	Scales	0.67 (±0.1)	6608 (±531)	4202 (±432)	374000	383 (±62)	2.38 (±0.21)	8 (±1.3)	931 (±93)	242 (±5)	24 (±2)	3 (±1)	0.03 (±0.005)
	Water	No data	2019	No data	2565	8	*0.089*	*1.2*	*1.8*	29	9	0.86	*0.01*
EA	Scales	2.44 (±0.3)	3650 (±461)	4355 (±632)	374000	349 (±40)	0.85 (±0.12)	6 (±0.9)	1533 (±181)	240 (±5)	51 (±5)	59 (±14)	0.07 (±0.013)
	Water	No data	4519	No data	43013	65	*0.379*	2.8	77.4	146	45	1.58	0.49
LF	Scales	1.7 (±0.3)	4500 (±592)	4438 (±1152)	374000	320 (±29)	0.74 (±0.07)	5 (±1.1)	978 (±170)	306 (±8)	31 (±2)	6 (±1)	0.02 (±0.004)
	Water	No data	703	No data	690	9	*0.452*	2.8	2.9	11	21	0.50	*0.01*
ML	Scales	1.12 (±0.1)	4214 (±310)	4188 (±1735)	374000	295 (±19)	0.57 (±0.06)	5 (±2)	764 (±92)	336 (±9)	43 (±5)	4 (±1)	0.02 (±0.009)
	Water	No data	1469	No data	1337	10	*0.238*	2.3	2.6	20	15	*0.16*	*0.01*
MN	Scales	1.79 (±0.3)	4479 (±319)	4036 (±716)	374000	303 (±37)	0.66 (±0.07)	7 (±2.2)	782 (±99)	248 (±4)	35 (±3)	8 (±3)	0.05 (±0.019)
	Water	No data	1550	No data	1766	7	*0.408*	3.3	2.9	23	18	0.27	0.01
MW	Scales	2.76 (±0.4)	5214 (±547)	2943 (±391)	374000	166 (±25)	0.56 (±0.08)	8 (±1.7)	678 (±146)	325 (±7)	17 (±2)	9 (±3)	0.05 (±0.010)
	Water	No data	5575	No data	24923	12	*0.281*	3.9	5.2	147	19	3.18	0.08
SB	Scales	1.5 (±0.2)	4487 (±421)	3024 (±377)	374000	127 (±11)	0.66 (±0.05)	5 (±1.3)	704 (±52)	248 (±6)	49 (±3)	5 (±1)	0.07 (±0.006)
	Water	No data	44510	No data	80683	30	*0.496*	2.4	2.5	316	160	0.03	2.84
ANOVA and Kruskal-Wallis* df=11,260		F=17.09 *P*<0.001	F=3.07 *P*=0.001	F=2.01 *P*=0.028	N.A.	F=28.66 *P*<0.001	F=16.41 *P*<0.001	F=2.16 *P*=0.017	*H=52.55 *P*<0.001	F=128.99 *P*<0.001	F=44.55 *P*<0.001	*H=148.62 *P*<0.001	F=10.11 *P*<0.001
No. of significant Post Hoc* pairs		18	0	0	N.A.	32	11	0	9	48	33	27	12

Table 3. Cross validation LDFA classification of fish to their site of origin using all elements which showed significant differences in concentration in scales of *Salmo trutta* among sites (Table 2). *Shows the percentage of individuals correctly classified to their site of origin. Correctly classified individuals are shown in bold.

	No. of fish classified to each site based on element concentrations in scales of *Salmo trutta*													
Site	AB	AU	AD	CW	CD	CR	EA	LF	MN	ML	MW	SB	n	Correct* (%)
AB	**16**	0	0	0	1	2	1	0	0	1	2	0	23	70
AU	0	**28**	1	0	0	0	0	0	0	0	0	0	29	97
AD	0	0	**18**	0	0	0	1	0	0	0	0	1	20	90
CW	0	0	0	**20**	0	0	0	0	2	0	0	0	22	91
CD	0	0	0	0	**20**	1	0	0	0	1	0	0	22	91
CR	1	0	0	0	1	**19**	0	1	0	0	0	0	22	86
EA	0	0	2	0	0	0	**18**	1	0	0	0	0	21	86
LF	0	0	0	0	0	0	0	**12**	1	2	0	0	15	80
MN	0	0	0	0	0	0	1	0	**20**	0	0	0	21	95
ML	0	0	0	0	1	0	0	3	2	**14**	0	1	21	67
MW	1	0	0	0	0	1	0	1	0	1	**17**	0	21	81
SB	0	0	0	0	0	0	0	0	1	0	0	**23**	24	96

Table 4. Standardised Canonical Discriminant Function Coefficients for the LDFA performed using all elements in scales which exhibited significant differences in concentration between sites. The two most important elements in the first LDFA function are underlined and shown in bold.

	LDFA Function						
Element	1	2	3	4	5	6	7
Ba	0.79	-1.18	0.58	-0.40	0.04	0.00	0.09
Mg	**0.88**	0.20	0.45	0.17	0.12	0.10	0.23
Si	-0.20	0.15	-0.08	0.19	0.14	-0.09	-0.39
Mn	-0.37	0.50	0.61	0.79	-0.38	-0.30	0.05
Co	0.12	0.30	0.23	-0.19	0.65	0.55	0.23
Cu	-0.21	0.23	0.33	-0.24	0.08	-0.13	0.95
Zn	-0.12	0.04	0.03	0.04	0.08	0.59	-0.98
Sr	**-1.07**	-0.27	-0.45	0.11	0.36	0.01	0.02
Pb	0.47	-0.14	-0.69	1.20	0.28	0.08	0.24
U	-0.01	0.31	-0.45	-0.84	-0.46	0.06	-0.25
Li	-0.12	-0.08	-0.05	0.22	-0.58	0.63	0.44
Cumulative variance explained (%)	59	78	88	94	97	99	100

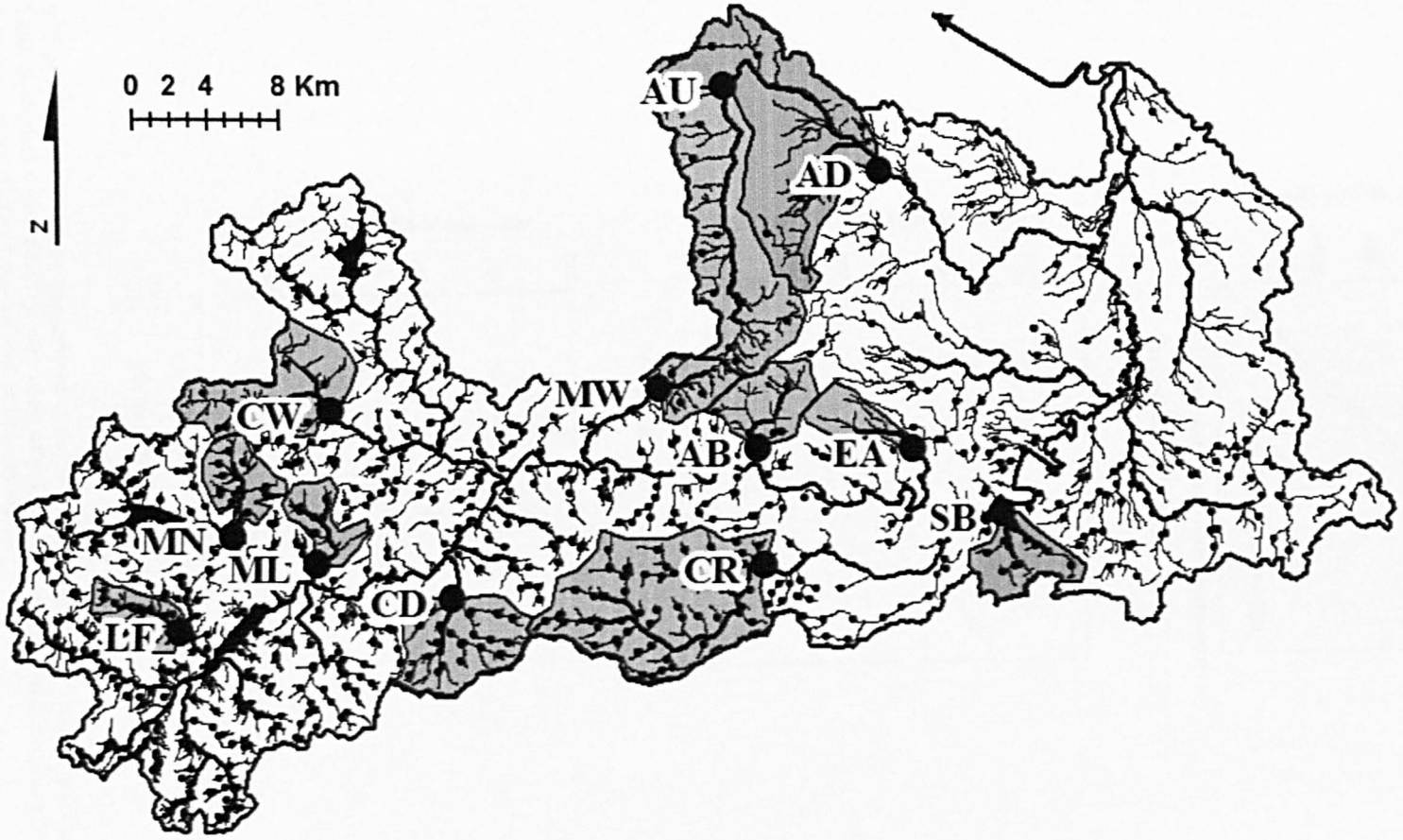
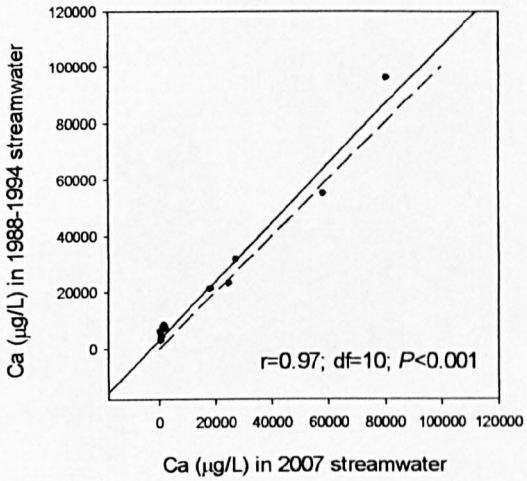
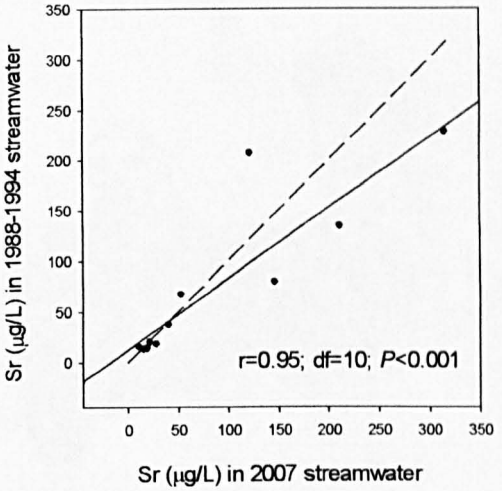


Figure 2. Map of Dee catchment showing the entire river network and the 1988-1994 British Geological Survey (BGS) stream water survey sites (small black circles) and 2007 survey sites (large black circles) with corresponding sub-catchment boundaries (grey areas). Site names are shown in Table 1.





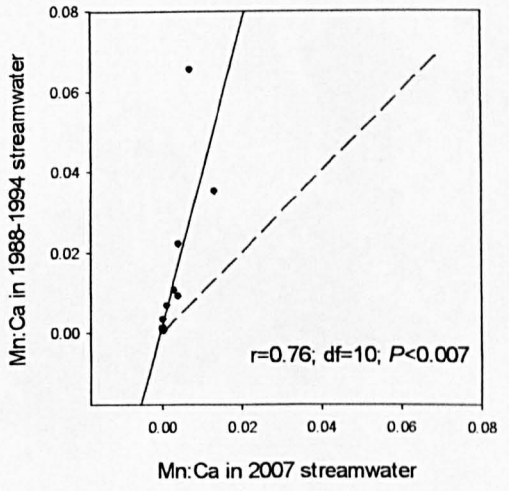


Figure 3. The correlation between concentrations of Ca, Sr and Mn:Ca in stream water in 2007 and in the 1988-1994 British Geological Survey (BGS) stream water data. The line of best fit (solid line) and the line denoting a 1:1 relationship between the two variables (dashed line) are shown.

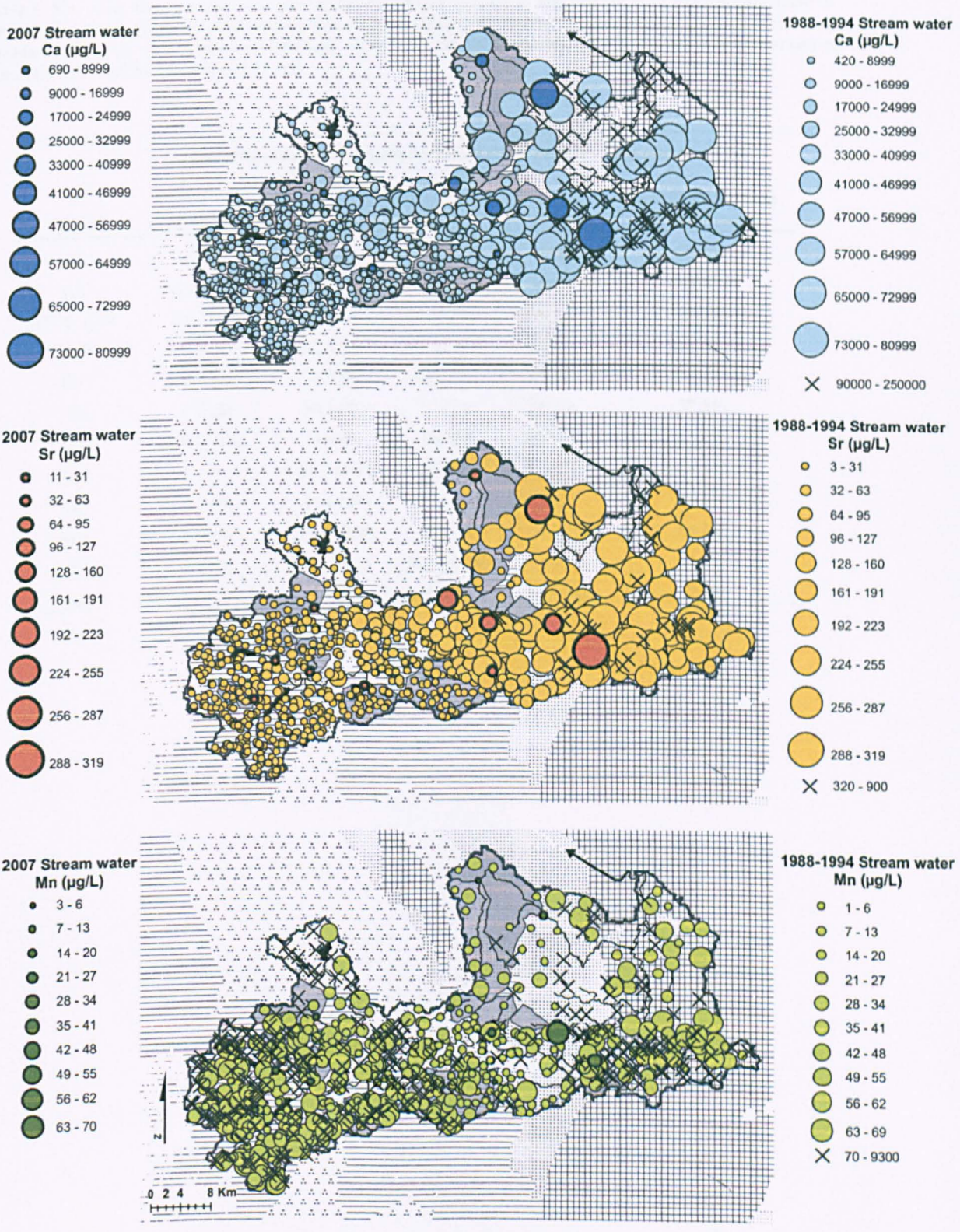


Figure 4. Concentrations of Ca, Sr, and Mn in 2007 stream water and 1988-1994 British Geological Survey (BGS) stream water. Concentrations of elements in 1988-1994 stream water were divided into two groups corresponding to the concentration range in the 2007 samples and those at higher concentrations (denoted with black crosses).

Table 5. Site code, site area (km^2) and proportion of bedrock type (% in brackets) in each site sub-catchment in the Dee catchment (Fig. 1). Data derived from British Geological Survey (BGS) bedrock geology data using ArcMap. *The AD was situated downstream of AU (on the same tributary) (Fig. 1) and so AD catchment boundary included the area drained by AU.

	Area of Site Sub-Catchments by Bedrock Geology Type [Area; km^2(%)]				
Site	**Carboniferous**	**Ordovician**	**Silurian**	**Triassic**	**Site Sub-Catchment Total Area**
AB	5.2 (25)	2.7 (13)	12.4 (61)	0.0 (0)	**20.35**
AU	33.5 (48)	6.5 (9)	30.3 (43)	0.0 (0)	**70.31**
AU & AD*	88.2 (73)	10.0 (4)	47.2 (23)	0.0 (0)	**70.31**
CD	0.0 (0)	32.4 (98)	0.54 (2)	0.0 (0)	**32.96**
CR	0.0 (0)	70.5 (84)	12.8 (15)	0.0 (0)	**83.27**
CW	0.0 (0)	30.1 (80)	7.1 (19)	0.0 (0)	**37.21**
EA	11.7 (100)	0.0 (0)	0.0 (0)	0.0 (0)	**11.69**
LF	0.0 (0)	21.7 (100)	0.0 (0)	0.0 (0)	**21.68**
ML	0.0 (0)	11.0 (98)	0.2 (2)	0.0 (0)	**11.25**
MN	0.0 (0)	15.8 (100)	0.0 (0)	0.0 (0)	**15.84**
MW	0.0 (0)	0.1 (0.5)	12.8 (99.8)	0.0 (0)	**12.91**
SB	7.5 (44)	0.0 (0)	0.0 (0)	10.0 (56)	**17.33**

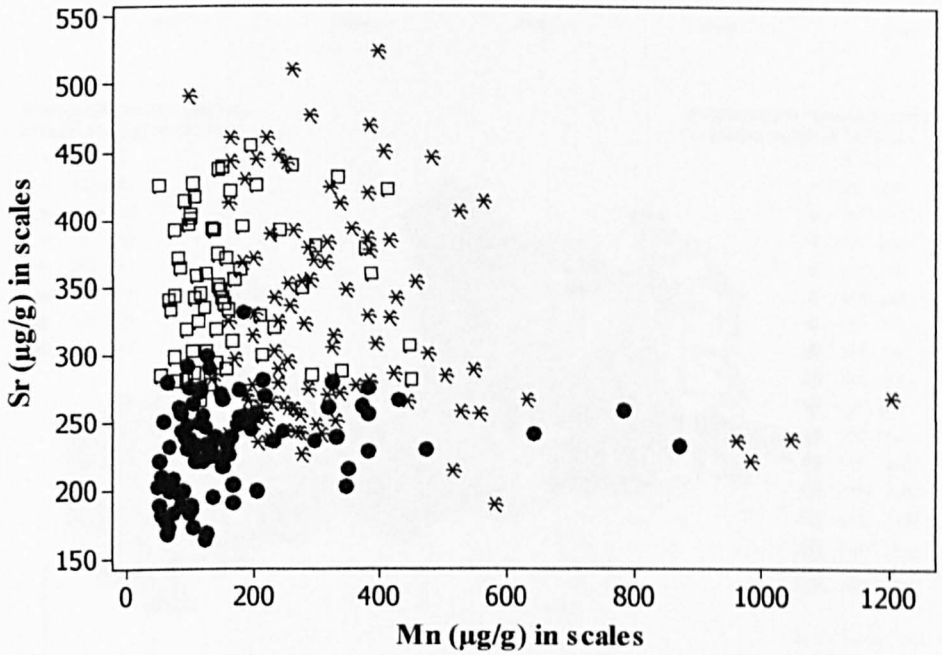


Figure 5. Scatter plot of Sr and Mn in scales of *Salmo trutta* in the Dee catchment. Scales from sites in the upper region (stars; sites LF, MN, CW, ML and CD), the middle region (open squares; sites CR, MW and AB,) and the lower region (solid circles; sites AU, AD, EA and SB) are shown.

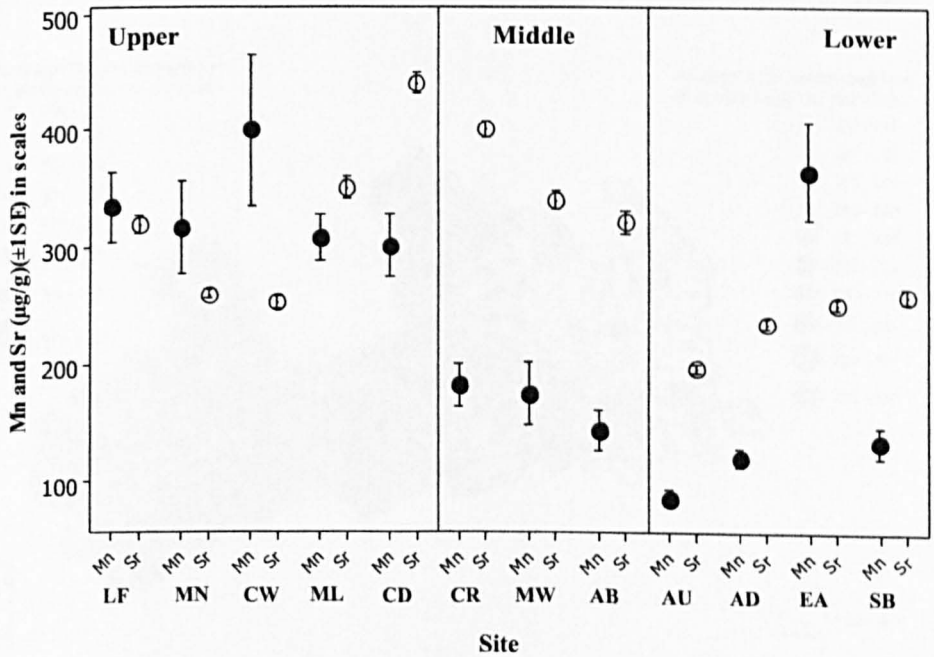


Figure 6. Mean concentrations of Sr (open circles) and Mn (solid circles) in scales of *Salmo trutta* at 12 sites in the Dee catchment (µg/g±1SE). Sites are ordered from the upper to the lower region of the catchment (Fig. 1). The plot area is divided into sections corresponding to sites in the upper, middle and lower regions of the catchment.

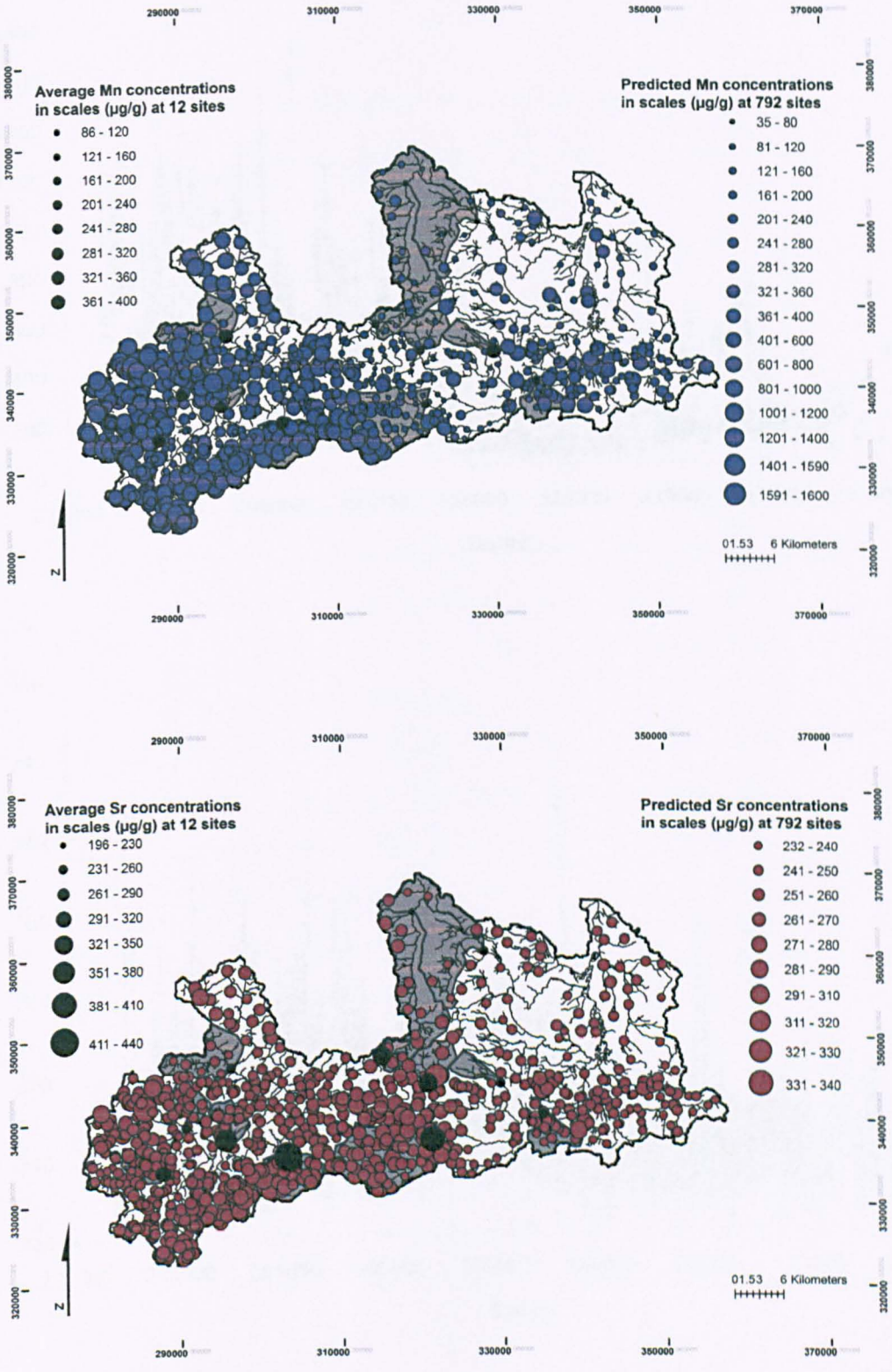


Figure 7. Predicted concentrations of Mn and Sr in scales of *Salmo trutta* (µg/g±1SE) in the Dee catchment at 792 sites, based on the relationship between element:Ca ratios in streamwater and scales.

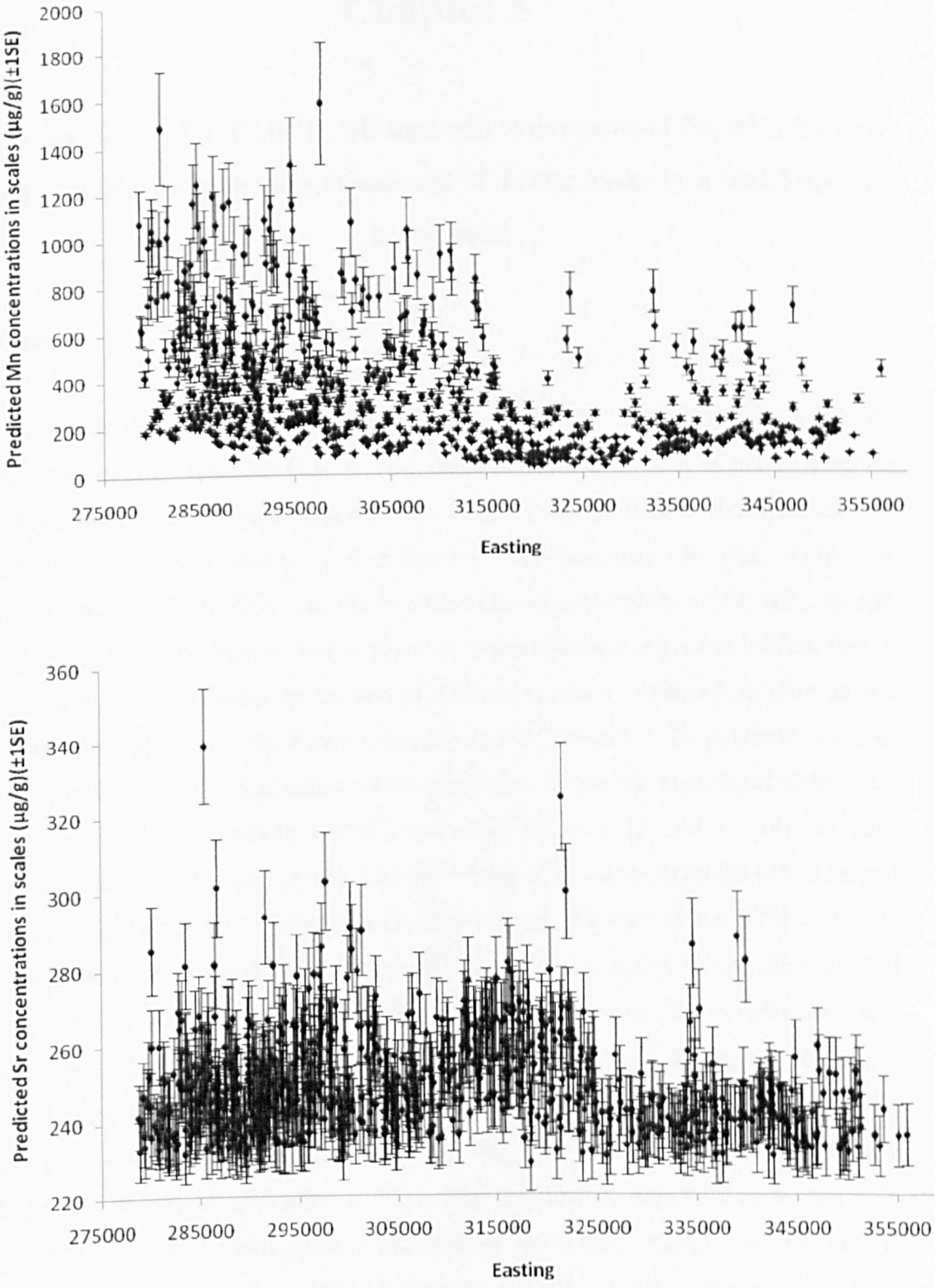


Figure 8. Predicted concentrations of Mn and Sr in scales of *Salmo trutta* (µg/g±1SE) in the Dee catchment at 792 sites, based on element:Ca ratios in streamwater (BGS data). Sites are ordered corresponding to longitudinal position.

Chapter 5

Comparison of $\delta^{15}N$, $\delta^{13}C$, δD and concentrations of Sr, Mn, Ba and Mg in scales as biogeochemical tags of *Salmo trutta* in a small upland catchment

Abstract

Natural variability in stable isotope ratios (e.g. $^{87}Sr:^{86}Sr$, $\delta^{18}O$, δD, $\delta^{15}N$ and $\delta^{13}C$) and element concentrations (e.g. Sr, Mn, Ba and Mg) in structures of fish (e.g. scales and otoliths) have provided biogeochemical tags of the spatial locations inhabited by individuals in both marine and freshwater environments. In this study, the performance of $\delta^{15}N$, $\delta^{13}C$ and δD in scales and concentrations of Sr, Mn, Ba and Mg in scales and otoliths of *Salmo trutta* as biogeochemical tags of individual fish in a small upland catchment are compared. Despite regional variability in altitude and rainfall throughout the catchment, no significant differences in δD in whole scales of *Salmo trutta* were detected among the 6 study sites. However, significant differences in $\delta^{15}N$ and $\delta^{13}C$ in whole scales were found between 12 and 13 pairs of sites respectively (out of a total of 15). Mean $\delta^{15}N$ and $\delta^{13}C$ among sites (‰±1SE) ranged between 9.5(±0.2) to 13.4(±0.1) and -24(±0.2) to -19.4(±0.1) for $\delta^{15}N$ and $\delta^{13}C$ respectively. The natural variability in $\delta^{15}N$ and $\delta^{13}C$ in scales among sites enabled fish to be classified to their site of origin with 93% accuracy. This classification was superior to that achieved when using Sr, Mn, Ba and Mg in the hydroxyapatite of scales (classification accuracy of 88%) or aragonite of otoliths (classification accuracy of 89%) from the same fish. Combining $\delta^{15}N$ and $\delta^{13}C$ and Sr, Mn, Ba and Mg improved the classification to 96%. The findings of this study show that $\delta^{15}N$ and $\delta^{13}C$ in scales could provide non-lethally collectable biogeochemical tags of *Salmo trutta* in a small upland catchment, superior in performance to element concentrations in otoliths and scales. Identifying the site of origin of migratory *Salmo trutta* smolts could be used to determine the relative success of different rearing habitats for producing migratory contingents of the species in freshwater.

Introduction

Natural variability in stable isotope ratios have offered a useful ecological tool for studying food-web relationships (discussed in Gannes *et al.*, 1997; DeNiro & Epstein, 1981) and for providing biogeochemical tags of individuals/populations (reviewed in Hobson, 1999). The ratio of ^{15}N to ^{14}N is usually expressed as delta (δ) notation, which is defined as parts per thousand (‰) deviations from an international standard ($\delta^{15}N$) (see Appendix A for background information on stable isotopes in ecology). $\delta^{15}N$ can be enriched from prey to predator (DeNiro & Epstein, 1981) (typically by 2‰-5‰) and can therefore provide an indication of the trophic level at which an animal feeds (Wainright *et al.*, 1993). The $\delta^{15}N$ at the base of a food web can vary significantly among spatial locations depending on the relative contribution of sources of nitrogen into a system (e.g. N_2 fixation, atmospheric deposition and anthropogenic input from fertilisers or waste water) (Montoya, 2007). The ratio of ^{13}C to ^{12}C (usually expressed as $\delta^{13}C$) remains relatively uninfluenced by trophic level feeding (trophic enrichment can be <1‰) but can vary among geographical locations due to variable C sources in the foodweb (e.g. benthic algae *vs.* allochthonous plant material, the concentration of dissolved inorganic C available to algae, the prevalence of C3 and C4 plants in the watersheds and the geologic contribution of C to the isotopic composition) (Kennedy *et al.*, 2005).

In addition to revealing food web relationships, stable isotope ratios have provided a useful stock discrimination tool for revealing the spatial ecology of fish species. For instance, natural gradients in $\delta^{13}C$ and $\delta^{15}N$ between freshwater (which tends to be relatively depleted in $\delta^{13}C$ and $\delta^{15}N$) and the marine environment (which tends to be relatively enriched in $\delta^{13}C$ and $\delta^{15}N$) have been used to distinguish between freshwater or marine feeding fish (reviewed in Hobson, 1999; e.g. Doucett *et al.*, 1999; Ciancio *et al.*, 2008). $\delta^{15}N$ in fish muscle has also revealed the fluvial-lacustrine migrations of fish (Maruyama *et al.*, 2001). Distinct $\delta^{13}C$ and $\delta^{15}N$ signatures in wild and farmed Atlantic salmon (*Salmo salar*) suggest that the technique might prove useful for identifying farm escapees (Dempson & Power, 2004). The technique has also been used to compare feeding patterns among populations of anadromous fish during marine life history stages (Dempson *et al.*, 2010). In a recent study, Sepulveda and co-authors (2009) used $\delta^{15}N$ in fish tissue to

distinguish between resident cutthroat trout (*Oncorhynchus clarkia utah*) inhabiting headwaters within the Bear River system in the U.S.A. and semi-migratory contingents of the species inhabiting fluvial river channels further downstream (Sepulveda *et al.*, 2009). $\delta^{15}N$ in fish muscle varied by ~4‰ between fish from headwaters and downstream sites (Sepulveda *et al.*, 2009). This was attributed in part to an influx of anthropogenic sources of nitrogen which enriched the $\delta^{15}N$ in stream water with increasing distance downstream when compared with the relatively pristine headwaters (Sepulveda *et al.*, 2009). The degree to which $\delta^{15}N$ might vary among downstream environments (e.g. among 3rd order tributaries) within a catchment was not examined (Sepulveda *et al.*, 2009) but might be expected to differ. For instance, there have been a number of studies which have found a positive correlation between the proportion of agricultural land in a catchment and enrichment of $\delta^{15}N$ in fish muscle (Kennedy *et al.*, 2005; Vandermyde & Whitledge, 2008), scales (Harrington *et al.*, 1998) and otoliths (Vandermyde & Whitledge, 2008). It has been possible to differentiate the stream of origin of fish within a catchment, based on $\delta^{13}C$ and $\delta^{15}N$ in fish (Harrington *et al.*, 1998; Schmetterling & Dawson, 2002), indicating the potential that natural variability in stable isotope ratios has as a stock discrimination tool in freshwater.

Spatial variability in the ratios of $^{2}H:^{1}H$ is usually expressed as delta (δ) notation, which is defined as parts per thousand (‰) deviations from an international standard (δD). δD in biological tissue has been used to study the migration patterns of species at a continental scale in terrestrial systems (reviewed in Hobson, 1999) and has recently shown promise as a tool for distinguishing the source locations of fish in freshwater (Whitledge *et al.*, 2007). Whitledge and co-authors found δD in otoliths of fish reflected differences in the evaporative fractionation of δD in water between lentic and lotic environments (Whitledge *et al.*, 2007). This allowed fish originating from these habitats to be distinguished based on δD in otoliths (Whitledge *et al.*, 2007). Variability in δD in terrestrial environments has also been attributed to latitudinal and altitudinal patterns in temperature and precipitation (Hobson, 1999; Darling & Talbot, 2003). δD in rainfall can be influenced by fractionation during evaporation and condensation in the atmosphere (Darling & Talbot, 2003). During condensation, raindrops become more enriched in the heavier isotope (^{2}H). As temperature drops and rainfall increases, raindrops become progressively more isotopically depleted as the total proportion of heavier isotopes in

the atmosphere decreases (Darling & Talbot, 2003). Detectable differences in δD have been found in precipitation in the order of 1.6‰ per $100m^{-1}$ change in altitude at low latitudes and 4.8‰ per $100m^{-1}$ change in altitude at high latitudes (Dansgaard, 1964; Ziegler, 1988). It is possible that variations in δD might occur over small spatial scales in fresh water providing that there is a sufficient change in altitude among locations to induce variability in rainfall. Therefore, in combination with other biogeochemical markers, δD might help to improve the discrimination of fish among geographical locations in freshwater.

Salmo trutta is an excellent candidate species for studies on biogeochemical tags, due to the continuum of life history strategies that the species exhibits, which can involve migration/movement over considerable distances (Ovidio *et al.*, 1998). The species is phenotypically plastic and can reside in freshwater during its entire lifespan or utilise a combination of freshwater, estuarine and marine habitats (Cucherousset *et al.*, 2005). This study examines the application of non-lethally collectable biogeochemical tags to elucidate the natal origins and movement patterns of the species during its freshwater life stages. $\delta^{15}N$ and $\delta^{13}C$ have been used to distinguish between freshwater-resident and migratory contingents of *Salmo trutta* (Ciancio *et al.*, 2008), for identifying the progeny of freshwater-resident and migratory *Salmo trutta* (McCarthy & Waldron, 2000) and for determining trophic level feeding (Grey, 2001). In this study we aim to determine the accuracy with which *Salmo trutta* can be classified to their site of origin in fresh water, based on naturally occurring stable isotope ratios in scales compared with element concentrations in scales of the same fish. Identifying the tributary of origin of *Salmo trutta* smolts could be used to reveal the relative success of different rearing habitats for producing migratory contingents of the species in fresh water. There do not appear to be any other studies which have explored variability in $\delta^{15}N$, $\delta^{13}C$ and δD in fish at the spatial resolution used in this study. While the relative contribution of different stable isotope ratios (specifically $\delta^{15}N$ and $\delta^{13}C$, and $^{87}Sr:^{86}Sr$) to discriminate fish among different locations has been examined (Dubé *et al.*, 2005; Kennedy *et al.*, 2005), quantifying the relative discriminatory power of element concentrations and stable isotope ratios has only been quantified in one study in the marine environment (Thorrold *et al.*, 1998) which is surprising given that in recent years a combination of element concentrations and stable isotope ratios have proved popular as biogeochemical tags of fish (Comyns *et al.*, 2008; Walther & Thorrold,

2008). Thorrold and co-authors (1998) found that analysing $\delta^{13}C$ and $\delta^{18}O$ in combination with Mg, Mn, Sr and Ba in otoliths increased the accuracy of assigning juvenile weakfish (*Cynoscion regalis*) to natal estuarine areas. The relative contribution of stable isotope ratios and element concentrations as biogeochemical tags is yet to be determined for fish among freshwater locations.

Muscle tissue is often used in studies examining variability in stable isotope ratios in fish. However, a number of studies have shown that $\delta^{13}C$ and $\delta^{15}N$ in fish muscle is highly correlated with $\delta^{13}C$ and $\delta^{15}N$ in non-lethally collectable calcified structures of the same fish, such as scales (Satterfield & Finney, 2002; Schmetterling & Dawson, 2002; Perga & Gerdeaux, 2003; Kennedy *et al.*, 2005; Kelly *et al.*, 2006). Regarding δD, it has been demonstrated that δD in otolith protein is highly correlated with δD in ambient water (Whitledge *et al.*, 2006) and one might also expect this to be true for fish scale collagen. The objectives of this study are to; i) examine variability in naturally occurring $\delta^{15}N$, $\delta^{13}C$ and δD in *Salmo trutta* scales over small spatial scales (among sites which are >7.5km apart) in freshwater and; ii) quantify the degree to which these naturally occurring stable isotope ratios might provide a biogeochemical tag of site of origin of *Salmo trutta* when compared with element concentrations in scales and otoliths of the same individuals.

Methods

The study was carried out in the Dee catchment in Wales (drainage area ~1800km^2), a major salmonid catchment in the U.K. which has freshwater-resident contingents of *Salmo trutta* in addition to an annual estimate of *c.* 9,300 sea-run *Salmo trutta* (for the period 1992-2003) (Davidson *et al.*, 2007). The headwaters are in the West of the catchment and drain an area with upland topology (maximum altitude: 800m). The catchment becomes more lowland in character towards the East of the catchment and the river discharges into the Irish Sea at Liverpool Bay. Rainfall varies considerably across the catchment. The upland areas receive an average annual rainfall of between 1800-2200mm whereas the lowland areas receive between 600-1100mm [based on annual rainfall data for the period 1971-2000 (Met Office, 2009)]. An estimated δD gradient in groundwater from *c.* -42‰ in the West of the catchment to *c.* -50‰ in the

East of the catchment might be expected based on interpolated data on δD in groundwater at sites in the region (Darling *et al.*, 2003).

Land use in the Dee catchment ranges from nutrient-poor grassland and forest (typically in the upland areas to the West of the catchment), to arable and improved grassland (typically in the lowland areas to the East of the catchment). Variability in N input from anthropogenic sources (e.g. fertilisers and waste water input) which might occur between such land use types might generate detectable differences in $\delta^{15}N$ in the catchment over small spatial scales (Harrington *et al.*, 1998).

The degree of variability in stable isotope ratios in freshwater fish among geographical locations, is often examined in fish collected from one site in each of several major tributaries within a catchment (Kennedy *et al.* 1997; Harrington *et al.*, 1998; Maruyama *et al.*, 2001; Kennedy *et al.*, 2005). *Salmo trutta* were collected during August and September 2007 from 1 site in each of 6 major tributaries in the Dee (Fig. 1). All fish included in the study were identified as freshwater-resident brown trout based on morphological features. The fish were caught by electrofishing using an InteliSYS Fish Magnet Mobile 11 (FMM) back-pack and euthanised on site using a U.K. Home Office approved Schedule 1 Method. A size range of fish were collected at each site (Schmetterling & Dawson, 2002) in order to determine whether there were relationships between fish size and stable isotope ratios in scales of *Salmo trutta*. The fish carcasses were frozen on return to the laboratory and stored at -24°C.

Fish were partially defrosted and scale samples were removed using plastic probes. Scales were manually cleaned in deionised (DI) water under a dissecting microscope using nylon fibre brushes and plastic tipped forceps. Scale samples were then ultrasonically cleaned and tripled-rinsed in DI water and allowed to dry in a laminar flow hood. All equipment in contact with the scales was acid-washed before use. Following methods reported elsewhere, we did not acid soak the scales prior to analysis (Kelly *et al.*, 2006). The hydroxyapatite component of scales contains C which is usually depleted in ^{13}C compared to collagen (Perga & Gerdeaux, 2003). However, Hutchinson & Trueman (2008) estimated that the effect of this source of C on $\delta^{13}C$ in scales was likely to be below analytical errors [typical analytical errors were reported as *c.* 0.2-0.3‰ (Hutchinson & Trueman, 2006)]. In support of this, a study by Sinnatamby and co-authors (2007) which compared $\delta^{15}N$ and $\delta^{13}C$ in decalcified scales and non-decalcified scales of fish found no significant effect of

decalcification on $\delta^{15}N$ and $\delta^{13}C$. In any case, as the C in hydroxyapatite is probably derived from Dissolved Inorganic Carbon (DIC) (Perga & Gerdeaux, 2003) which might vary among geographical locations, it could therefore contribute to the differences in $\delta^{13}C$ among sites in this study.

To obtain sufficient scale sample for analysis, multiple whole scales from each fish (between 3 and 20 depending on the size of the fish) were combined for each analysis (Kennedy *et al.*, 2005). 1.5mg (±0.1 SD) of scales from each fish were encapsulated in tin cups (Elemental Microanalysis Ltd, U.K.) and were analysed for C and N stable isotope ratios using a PDZ Europa Scientific Roboprep elemental analyzer coupled to a PDZ Europa Hydra 20-20 stable isotope ratio mass spectrometer (Sercon Ltd., UK) at the University of California, Davis, Stable Isotope Facility (UC Davies SIF), U.S.A.

There was sufficient scale material available to determine δD for 84 of the 89 fish included in the study (Table 1). For δD analysis, 0.53(±0.04SD)mg of scale sample from each fish, which had been prepared in the manner described above, was encapsulated in silver cups (Elemental Microanalysis Ltd, U.K.) for analysis using a Heckatech HT Oxygen Analyzer coupled to a PDZ Europa 20-20 isotope mass spectrometer (Sercon Ltd., UK) at the UC Davies SIF (U.S.A). Hydrogen forms weak bonds with nitrogen and oxygen and can exchange with ambient water vapour (Schimmelmann, 1991). While it is possible to extract the exchangeable hydrogen (Wasenaar & Hobson, 2000), this is not necessary, providing that all samples within a study are prepared and analysed in the same environment (Hobson, 1999) or at least allowed to equilibrate with ambient laboratory water vapour prior to analysis.

Isotope ratio values are expressed as delta (δ) notation, which is defined as parts per thousand (‰) deviations from the international standards PeeDee Belemite (PDB) for carbon, Air for nitrogen and Vienna Standard Mean Ocean Water (VSMOW) for hydrogen. The isotope ratios are calculated by the following equation: $X = [(R_{sample} - R_{standard})/ R_{standard}] \times 1000$, where X is $\delta^{15}N$, $\delta^{13}C$ or δD and R is the ratio of the heavy isotope to the light isotope (^{13}C:^{12}C, ^{15}N:^{14}N or ^{2}H:^{1}H) in the sample and the reference material. To determine analytical precision, standards were analysed approximately every 13 samples. To examine repeatability, 4 replicate scale samples were analysed from each of 3 fish of a range of sizes (125, 187 and 206mm in fork length (FL)). The SD of these replicates was <0.1‰ for $\delta^{15}N$, $\delta^{13}C$. To examine repeatability in δD, 4 replicate scale samples were analysed from each

of 3 fish of a range of sizes (139, 206 and 257mm in fork length (FL)). The SDs of these replicates were 2.5‰, 2.9‰ and 5.12‰ respectively.

$\delta^{15}N$, $\delta^{13}C$ of δD might vary with fish size/age due to different trophic level feeding or due to variable metabolic fractionation (Acolas *et al.*, 2007; Ciancio *et al.*, 2008; Etheridge *et al.*, 2008). In this study, fish fork length was used to examine the effect of fish size/age on stable isotope ratios. ANCOVA was used to reveal common within-group regression slopes of stable isotope ratios and fork length at each site by using each stable isotope ratio in the scales as the response variable, site as a category variable and fork length as a covariate. Providing that the assumption of equal slopes across sites was met, the effect of fork length on stable isotope ratios could be removed by using the common within-group regression coefficient in the following equation, adapted here from Almeida *et al.* (2008):

$$AC_{ij} = UAC_{ij} - [\beta \times (Log_{10}FL_j - Log_{10}\underline{FL})]$$

where AC_{ij} is the adjusted transformed stable isotope ratios for stable isotope ratio i of the j specimen, UAC_{ij} is the unadjusted character measurement for stable isotope ratio i of the j specimen (UAC was $\log_{10}$ transformed), β is the equal common within-group regression coefficient of stable isotope ratio i ($\log_{10}$ transformed) regressed against fork length ($\log_{10}$ transformed), FL_j is the fork length of the j specimen ($\log_{10}$ transformed) and $\underline{FL}$ is the mean fork length of all fish ($\log_{10}$ transformed).

The ANCOVA was repeated (this time with the adjusted stable isotope ratio data as the response variable) to confirm that the regression slope between fork length and stable isotope ratios were no longer significant ($P>0.05$). Within-site regressions of adjusted stable isotope ratio data were used to confirm that length was no longer a significant covariate. The adjusted stable isotope ratio data values were used in all subsequent analysis. ANCOVA is robust to departures from normality providing data are homoscedastic (Olejnik & Algina, 1984) and it has been used to correct for size effects on non-normal data (Claytor *et al.*, 1991; Almeida *et al.*, 2008).

Once any effect of fish fork length on stable isotope ratios was removed, differences in stable isotope ratios among sites were compared using One-way Analysis of Variance (ANOVA). Scheffe's post hoc tests were used to reveal the

pairs of sites between which significant differences occurred. The data were tested for normality using Anderson-darling's test and tested for equal variance using Bartlett's test (if data were normally distributed) or Levene's test (if data were non-normally distributed).

The degree to which fish scales could be used to distinguish the geographical site of origin of *Salmo trutta* based on stable isotope ratio data was tested using Linear Discriminant Function Analysis (LDFA). Some of the variables (in this case stable isotope ratios) were not normally distributed. Minor deviations from normality do not preclude the use of LDFA (Leakey *et al.*, 2008). The LDFA involved an 'original' classification (which used the discriminant functions to classify the same samples that were used to develop the functions) and a 'cross-validation' (CV) classification (which involves leaving one sample out of the dataset before establishing the discriminant functions and then classifying the sample that was removed) (Sharma, 1996). Multinomial Logistic Regression (MLR) was used to validate the overall and group classifications achieved with the LDFA and to monitor potential effects of non-normality on the LDFA results. All LDFAs and MLRs in this study were carried out on only those fish for which both stable istope data and element concentrations data (Sr, Ba, Mn and Mg) in scales were available (n=80). Canonical Variate Plots provide a visual representation of the classification of individual fish to their site of origin. Cohen's Kappa statistic was used compute the chance-corrected agreement between actual and predicted group (site) memberships of fish (Titus *et al.*, 1984; Barnett-Johnson *et al.*, 2008). The Kappa statistic ranges between 0 (indicating the classification to site was no improvement over that achieved by chance) and 1 (indicating that there was perfect agreement in the classification to site when taking into account classification by chance).

The relationship between $\delta^{15}N$ in scales and land use in the sub-catchments for each site was examined by calculating the percentage area of arable land and improved grassland (as these landuse types might receive anthropogenic sources of N through the application of fertilisers) and correlating with this $\delta^{15}N$ in scales at each site. Data on land use in the region were provided by the Countryside Council for Wales (2008). N.B. No data on landuse was available for the sub-catchment for site SB.

Gradients in $\delta^{13}C$ have been found within freshwater tributaries. There is evidence that $\delta^{13}C$ can become more enriched with increasing distance downstream

(Rasmussen *et al.*, 2009). In order to determine whether isotopic gradients within each tributary were responsible for inter-site differences in stable isotope ratios, correlations were carried out between $\delta^{13}C$, $\delta^{15}N$ and δD in scales and the distance (km) to each site from its upstream source. Pearson's Product Moment Correlation Coefficient was used where data met assumptions of normality and Spearman's Rank Correlation Coefficient was used where not, denoted r_p and r_s respectively.

Existing data on concentrations of Sr, Mn, Ba and Mg in recently deposited hydroxyapatite of scales and aragonite of otoliths for 80 of the 89 fish included in this study were available (originally reported in Chapter 3). The scale and otolith sample preparation, analytical techniques, precision and accuracy are reported in Chapter 3. Briefly, one undamaged non-regenerated scale and one otolith per fish were rigorously cleaned manually and ultrasonically in 3% hydrogen peroxide. Element concentrations in the distal area of the hydroxylapatite layer of scales (an area corresponding to scale material deposited during the most recent summer of growth) were determined using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). Data on Sr, Ba, Mn and Mg concentrations were established as important discriminators of site of origin in the Dee catchment [data reported elsewhere (Chapter 3)]. The element concentration data are re-analysed here to allow a comparison to be made between the discrimination to site of origin achieved using stable isotope ratios and the discrimination achieved using element concentrations. Finally, the element concentration data and the stable isotope ratio data were entered into the LDFA simultaneously in order to assess their performance as combined biogeochemical tags of *Salmo trutta*.

All statistical analysis was carried out in SPSS (v. 16) and MINITAB (v. 14).

Results

Site locations and fork length of *Salmo trutta* collected in the Dee catchment in 2007 are shown in Figure 1 and Table 1 respectively.

$\delta^{15}N$, $\delta^{13}C$ and δD were tested for normality using Anderson-darling's test and tested for homogeneity of variances using Bartlett's test (if data were normally distributed) or Levene's test (if data were non-normally distributed). $\delta^{13}C$ values exhibited homogeneity but did not follow a normal distribution. A positive constant

corresponding to the lowest negative value plus 1 was added to the $\delta^{13}C$ data to allow $\log_{10}$ transformation. However, despite transformation the data were neither normally distributed nor homogeneous. Therefore untransformed $\delta^{13}C$ data were used in the remaining statistical analysis. The $\delta^{15}N$ data were not normally distributed or homogeneous. $\text{Log}_{10}\delta^{15}N$ data were not normally distributed but did exhibit homogeneity of variance. Graphical inspection of $\delta^{13}C$ and $\log_{10}\delta^{15}N$ data (McGuinness, 2002) revealed only minor deviations from normality and so were used in all remaining statistical analyses. The δD data were normally distributed and exhibited homogeneity of variance.

There was a significant difference in fish fork length among sites ($F_{5,88}$ = 3.40; P= 0.008). ANCOVA revealed weak but statistically significant equal common within-group regression slopes for fork length and δD (β=0.07; P<0.001). The correlation between length and δD data when pooled across all sites was significant (Fig. 2). The effect of length was removed (see Methods section) and the adjusted data used for all remaining statistical analysis. There was no significant equal common within-group regression slope for fork length and $\delta^{15}N$ or $\delta^{13}C$. However, when correlations were carried out at individual sites, significant correlations were found between $\delta^{15}N$ in scales and fish fork length at site CR (Fig. 3) and between $\delta^{13}C$ in scales and fish fork length at site AU (Fig. 4) only. There was no correlation between $\delta^{15}N$ and $\delta^{13}C$ values (r_s=-0.114; df=88; P=0.288) pooled across all sites. ANOVA revealed significant differences in $\log_{10}\delta^{15}N$ and $\delta^{13}C$ values among sites (P<0.001), but not δD values (P=0.903) (Table 2).

A scatter plot of $\delta^{13}C$ and $\delta^{15}N$ values indicated that scale samples have distinct stable isotope ratios among sites (Fig. 5). There was no correlation between $\delta^{15}N$ and the percentage cover of arable land (r_s=0.43; df=4; P=0.47) or between $\delta^{15}N$ and percentage cover of combined arable land and improved grassland (r_s=-0.40; df=4; P=0.51) (Table 1). There was no significant relationship between the downstream distance of each site (Table 1) and $\delta^{13}C$ (r_s=0.10, df=5, P=0.36), $\delta^{15}N$ (r_s=-0.16, df=5, P=0.13) or δD (r_p=0.10, df=5, P=0.33) in scales.

The distinct $\delta^{15}N$ and $\delta^{13}C$ signatures in scales of fish among sites (Table 1 and 2, Fig. 5) suggest that it might be possible to classify *Salmo trutta* to their site of origin based on $\delta^{15}N$ and $\delta^{13}C$ in scales.

When the LDFA was carried out on $\log_{10}\delta^{15}N$ data only, the cross-validation (CV) classification accuracy was 58% (Table 3). The LDFA was then repeated for

$\delta^{13}C$ only, and resulted in a CV classification accuracy of 63% (Table 3). The two variables appear to have similar discriminatory powers. To assess the combined discriminatory power of $\delta^{15}N$ and $\delta^{13}C$ data, the LDFA was repeated using both datasets and this improved the CV classification to 93% (Table 3 and 4).

Data on element concentrations in the hydroxylapatite of scales and aragonite of otoliths (originally reported in Chapter 3) were available for 80 of the 89 fish included in the study. When the data on Sr, Mn, Mg and Ba concentrations in scales and otoliths were used in the LDFA, the CV classifications were 88% and 89% respectively (Table 3), therefore providing a poorer classification of fish to site of origin when compared with $\delta^{15}N$ and $\delta^{13}C$ in scales of the same fish. Finally, the stable isotope data ($\delta^{15}N$ and $\delta^{13}C$) were combined with the element concentration data (Sr, Mn, Mg and Ba) in scales of the same fish and the LDFA was repeated. The CV classification improved to 96% (Table 3). LDFA classification accuracies at each site are shown in Table 4.

MLR was used to validate the classification accuracies determined by the LDFA. There was good agreement between the original LDFA and the MLR indicating that variables (stable isotope ratios and element concentrations) which failed the assumptions of the LDFA did not appear to influence classification results of the LDFA (Table 3).

Discussion

Biogeochemical tags have become a popular tool in fisheries research (Thorrold *et al.*, 1998; Comyns *et al.*, 2008; Walther & Thorrold, 2008). In recent years combining stable isotope ratios and element concentration data for individual fish has been used to improve the discriminatory power of the technique (Walther & Thorrold, 2008; Comyns *et al.*, 2008). The relative strength of various combinations of element concentrations and stable isotopes ratios has rarely been quantified, despite the potential implications that this might have for prioritising analysis of particular stable isotopes or element concentrations in fish structures when limited quantities of sample are available for individual fish (e.g. if non-lethally collectable scale samples or archived samples are used). While Thorrold *et al.* (1998) compared the relative performance of element concentrations (Mg, Mn, Sr and Ba) and stable

isotope ratios ($\delta^{13}C$ and $\delta^{18}O$) as biogeochemical tags of an estuarine species, this is the first study that has compared the relative performance of these types of biogeochemical tags in fish in freshwater.

The relative performance of biogeochemical tags in scales of the same fish was quantified in this study. When element concentrations (Sr, Mn, Ba and Mg) and stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) were used in the LDFA, the classification of fish to site of origin was 4% and 5% higher when using $\delta^{15}N$ and $\delta^{13}C$ data in scales when compared with Sr, Mn, Ba, Mg data in otoliths and scales respectively. This suggests that intra-site variability in element concentrations in scale hydroxyapatite and otolith aragonite is greater than $\delta^{15}N$ and $\delta^{13}C$ in scale collagen.

The use of whole scales for the stable isotope analysis provides an integrated signal of $\delta^{15}N$ and $\delta^{13}C$ in collagen deposited throughout the lifetime of fish that is biased towards the collagen formed later in life. However, had the most recently formed collagen been isolated for analysis by removing the outer growth regions of the scale using a scalpel (Hutchingson & Trueman, 2006), the LDFA classification accuracy of $\delta^{15}N$ and $\delta^{13}C$ might have been even higher. The removal of >1mg of scale sample from a juvenile salmonids could leave a fish particularly vulnerable to infection or parasitism (Schmetterling & Dawson, 2002). While the amount of sample removed for $\delta^{15}N$ and $\delta^{13}C$ analysis in this study was 1.5mg, it has been possible to analyse <0.8mg (Syväranta *et al.*, 2008) and ~0.4mg (C. Trueman, National Oceangography Centre (NOC), *pers. comm.*, 2009) of scale material for $\delta^{15}N$ and $\delta^{13}C$ determination. Strong positive correlations have been found between both $\delta^{15}N$ and $\delta^{13}C$ in scales and otoliths (Kennedy *et al.*, 2005) and so presumably analysis of otolith growth rings would provide a chronological record of $\delta^{15}N$ and $\delta^{13}C$ which might be used to retrospectively reveal movements of fish among locations in freshwater, providing that $\delta^{15}N$ and $\delta^{13}C$ signatures primarily reflected spatial movements of fish rather than dietary changes. However, the relatively small amount of N in otoliths might limit the suitability of N in otolith growth bands for deriving a chronological history of $\delta^{15}N$ for individual fish due to analytical constraints (Harrington *et al.*, 1998).

This is the first study that has examined δD in fish scales. Despite considerable differences in altitude and rainfall between the West and East areas of the Dee catchment, we found no significant differences in δD in scales among sites. This may have been due to relatively poor repeatability. Poor repeatability might be

due to the fact that no correction was made for the portion of exchangeable hydrogen in the samples. In contrast to Whitledge *et al.* (2006) we found a relationship between fish fork length and δD in scales. A multi-species comparison of both terrestrial and aquatic organisms found that there was a correlation between trophic level feeding and δD (Birchall *et al.*, 2005). However, in the current study it seems unlikely that the relationship between δD and fish length was due to dietary changes because no correlations were found between $\delta^{15}N$ and fish length at most sites. The positive correlation between δD in scales and fish length in this study could be due to differences in the proportion of exchangeable hydrogen in scales equilibrating with ambient water vapour. The exchangeable portion of hydrogen (i.e. non-carbon-bound hydrogen) is thought to constitute ~25% of the hydrogen in collagen (Birchall *et al.*, 2005). Not all of the exchangeable hydrogen in scales in this study would have been exposed to ambient water vapour. As scales increase in size, the surface area to volume ratio presumably decreases, therefore reducing the relative amount of hydrogen in contact with ambient water vapour. Our scale samples were analysed whole (i.e. neither ground or cut into equal sized pieces) and so the relative proportion of exchangeable hydrogen in contact with ambient water vapour would have decreased with increasing fish/scale size. In the study by Whitledge *et al.*, (2006), in which no corrections were made for exchangeable hydrogen, no significant correlation was found between fish length and δD in muscle and otoliths (Whitledge *et al.*, 2006). This may be because the samples were ground which would allow equal equilibration of exchangeable hydrogen in the sample with ambient water vapour irrespective of whether the surface area of the original samples of muscle/otolith varied.

While relatively few studies have examined $\delta^{13}C$ and $\delta^{15}N$ among populations of wild freshwater-resident *Salmo trutta*, $\delta^{15}N$ and $\delta^{13}C$ values are known to be more enriched in anadromous compared with freshwater-resident *Salmo trutta* (Acolas *et al.*, 2007; Etheridge *et al.*, 2008) and the relationship between fish length and $\delta^{15}N$ in fish tissues has been used to indicate dietary changes in andromous *Salmo trutta* (Grey, 2001; Acolas *et al.*, 2007; Etheridge *et al.*, 2008). While there was a significant correlation between $\delta^{15}N$ and fish length at site CR, at other sites freshwater-resident *Salmo trutta* appear to forage at a similar trophic position. While this has been documented elsewhere for muscle $\delta^{15}N$ and fish length (Maruyama *et al.*, 2001), in this study the lack of correlation between $\delta^{15}N$ and fish

length at some sites might reflect the fact that whole scales have provided an integrated signal of $\delta^{15}N$ in collagen material formed throughout the lifetime of fish rather than an isotopic signature reflecting only recent feeding practices.

The range in $\delta^{15}N$ and $\delta^{13}C$ among sites in the Dee was similar to the range reported in another study carried out in freshwater (Kennedy *et al.*, 2005). This is particularly interesting considering that the study was carried out over a small spatial scale (drainage area of the Dee catchment ~1800km^2 and distances between neighbouring sites >7.5km). Variability in $\delta^{15}N$ among sites is probably due to exogenous factors such as spatial differences in land use (Kennedy *et al.*, 2005; Harrington *et al.*, 1998) although no relationship between $\delta^{15}N$ and the proportion of arable/improved grassland in the areas draining to each site was found in this study.

Kennedy *et al.*, (2005) attributed differences in $\delta^{13}C$ among freshwater sites to variable C sources in the food web (e.g. benthic algae *vs.* allochthonous plant material, the concentration of dissolved inorganic C available to algae, the prevalence of C3 and C4 plants in the watersheds and the geologic contribution of C to the isotopic composition). The range of $\delta^{13}C$ values observed in this study was surprisingly broad, almost spanning values reported for freshwater fish [$\delta^{13}C$ in muscle: -27.2±0.9 (mean±SE)] (Etheridge *et al.*, 2008) and those reported for North East Atlantic marine fish ($\delta^{13}C$ in muscle; -17.8±0.3 (mean±SE)) (summarised from the literature in Etheridge *et al.*, 2008) (Fig. 5).

$\delta^{13}C$ is generally found to be enriched in scales compared with muscle. Some reported values for the degree of enrichment of scales compared with muscle are: 1-2‰ (Schmetterling & Dawson, 2002), 2-4‰ (Kennedy *et al.*, 2005), 3‰ (Pruell *et al.*, 2003; Syväranta *et al.*, 2008), 3.7‰ (Satterfield & Finney, 2002) and 4‰ (Perga & Gerdeaux, 2003). Hutchinson & Trueman (2006) reported a range of $\delta^{13}C$ values from -17.4‰ to -14.5‰ in scale collagen of Atlantic salmon (*Salmo salar*) formed during marine residency. The most enriched $\delta^{13}C$ value for scales recorded in this study was -18.87‰ at site CR. This value is fairly close to the lowest value for $\delta^{13}C$ in the Atlantic salmon scales (-17.4‰) which might indicate fish feeding in an estuarine environment. If the fish at site CR had migrated to the estuary to feed then Sr concentrations in the scale hydroxyapatite of these fish should also be relatively high. The Sr concentrations measured in the scales and otoliths from fish site CR were some of the highest values recorded when compared with other sites in the Dee catchment (Chapter 4, Table 2). However, the values were not as high as the Sr

concentrations measured in the scales of sea trout which had been residing in the marine environment (Chapter 2, Table 7). An alternative explanation for the high $\delta^{13}C$ values at site CR is that the fish are either feeding on stocked fish which have been fed a predominantly marine-based diet prior to release or that fish as this site are feeding on small anadromous fish which have returned to freshwater tributaries after a period of marine or estuarine feeding (Etheridge *et al.*, 2008). There was a positive correlation between $\delta^{15}N$ and fish length at site CR but the range of $\delta^{15}N$ at this site was between 9.64‰ and 10.74‰ which might be insufficient to indicate a trophic level shift. Piscivory has been documented in *Salmo trutta* >130mm in length (L'Abée-Lund *et al.*, 1992; Jonsson *et al.*, 1999, Grey, 2001) suggesting that the relatively high $\delta^{15}N$ in scales of fish 177mm and 198mm at site CR (Table 1) could be due to piscivory. Site Cr is known to generate sea-run *Salmo trutta* (sea trout) and Atlantic salmon (*Salmo salar*) which return to spawn in the tributary and so an alternative explanation is that $\delta^{13}C$ from dead anadromous fish are influencing baseline $\delta^{13}C$ at site CR. Certainly, an influx to freshwater environments of energy and nutrients from the inward migration of marine dwelling anadromous fish has been documented (Jonsson & Jonsson, 2003). It is also possible that the fish from this site were stocked and that the analysis of whole scales included some scale material formed during juvenile growth while the fish were in the hatchery and being fed on a marine-based diet (Kennedy *et al.*, 2005).

$\delta^{15}N$ and $\delta^{13}C$ in scales have been found to vary among sites within a tributary (Schmetterling & Dawson, 2002) and so further work should examine the degree to which $\delta^{15}N$ and $\delta^{13}C$ in scales might vary among sites within a tributary in the Dee catchment. Gradients in $\delta^{13}C$ have been found within freshwater tributaries, with $\delta^{13}C$ becoming more enriched with distance downstream (Rasmussen *et al.*, 2009). We found no systematic change in $\delta^{15}N$ and $\delta^{13}C$ values in scales with distance of sites from their headwater sources. While this does not confirm whether or not intra-tributary isotopic gradients exist in the Dee catchment, it does indicate that the differences in $\delta^{15}N$ and $\delta^{13}C$ were not solely due to the distance of sites downstream from the headwaters and indicates that the differences in $\delta^{15}N$ and $\delta^{13}C$ were probably due to differing baseline isotopic ratios in the Dee catchment. In order to determine the accuracy with which the geographical origin of fish could be determined based on $\delta^{15}N$ and $\delta^{13}C$ in scales, it would probably be necessary to

create a map of the the isotopic variation within the catchment (Schmetterling & Dawson, 2002).

Archived scale samples might provide useful biogeochemical tags for monitoring changes in productivity of different areas in a catchment for generating smolts depending on whether $\delta^{15}N$ and $\delta^{13}C$ are temporally stable among years in freshwater. This has been confirmed in a two year study on $\delta^{15}N$ in fish in a freshwater catchment (Harrington *et al.*, 1998). However, in some lacustrine environments $\delta^{15}N$ and $\delta^{13}C$ in scales have been found to vary among years (Perga & Gerdeaux, 2003; Gerdeaux & Perga, 2006). Further work needs to examine the temporal stability of $\delta^{15}N$ and $\delta^{13}C$ baselines among locations in a freshwater catchment.

This study shows that scales could offer a non-lethal biogeochemical tag of *Salmo trutta* for identifying geographical origin of out-migrating fish. While $\delta^{15}N$ and $\delta^{13}C$ signatures in scale collagen might be relatively stable compared with element concentrations in scale hydroxyapatite [as hydroxyapatite might undergo continued crystallisation (Fouda, 1979) or even post-depositional changes in composition once formed (Fouda, 1979; Wells *et al.* 2003; Chapter 2 and 3)], the stability of $\delta^{15}N$ and $\delta^{13}C$ once incorporated into fish structures should be examined (Kennedy *et al.*, 2005).

While dual $\delta^{15}N$ and $\delta^{13}C$ can be carried out on the same sample relatively quickly and inexpensively compared with other stable isotope ratios (Harrington *et al.*, 1998), consideration should be given to the analysis of other stable isotope ratios such as $\delta^{18}O$ (Rooker *et al.*, 2008), ^{87}Sr:^{86}Sr (Kennedy *et al.*, 1997) and $\delta^{34}S$ (Weber *et al.*, 2002) which have been shown to provide powerful biogeochemical tags of fish. Although $\delta^{18}O$ can vary spatially, given that it can be highly correlated with δD in the environment [because stable isotope ratios of these elements can be influenced in the same way by condensation and evaporative fractionation (Darling & Talbot, 2003; Darling *et al.*, 2003), it is likely that $\delta^{18}O$ probably would not offer a useful biogeochemical tag of *Salmo trutta* in the Dee catchment, because δD did not vary significantly among sites in the Dee catchment.

The heterogeneous geology in the Dee catchment [ranging from Ordovician and Silurian to Carboniferous and Triassic (Chapter 4)] might generate differences in ^{87}Sr:^{86}Sr in streamwater (and therefore otolith aragonite and scale hydroxyapatite) over relatively small spatial areas. ^{87}Sr:^{86}Sr in otoliths has been successful in

revealing movement patterns of fish in large watersheds, such as the Ganges (>163,000km^2)(Milton & Chenery, 2005). The Dee catchment (~1800km^2), is a much smaller catchment with many contrasting lithologies (Chapter 4) and might provide an opportunity to examine fish origins/movements in a small catchment using ^{87}Sr:^{86}Sr in fish calcified structures.

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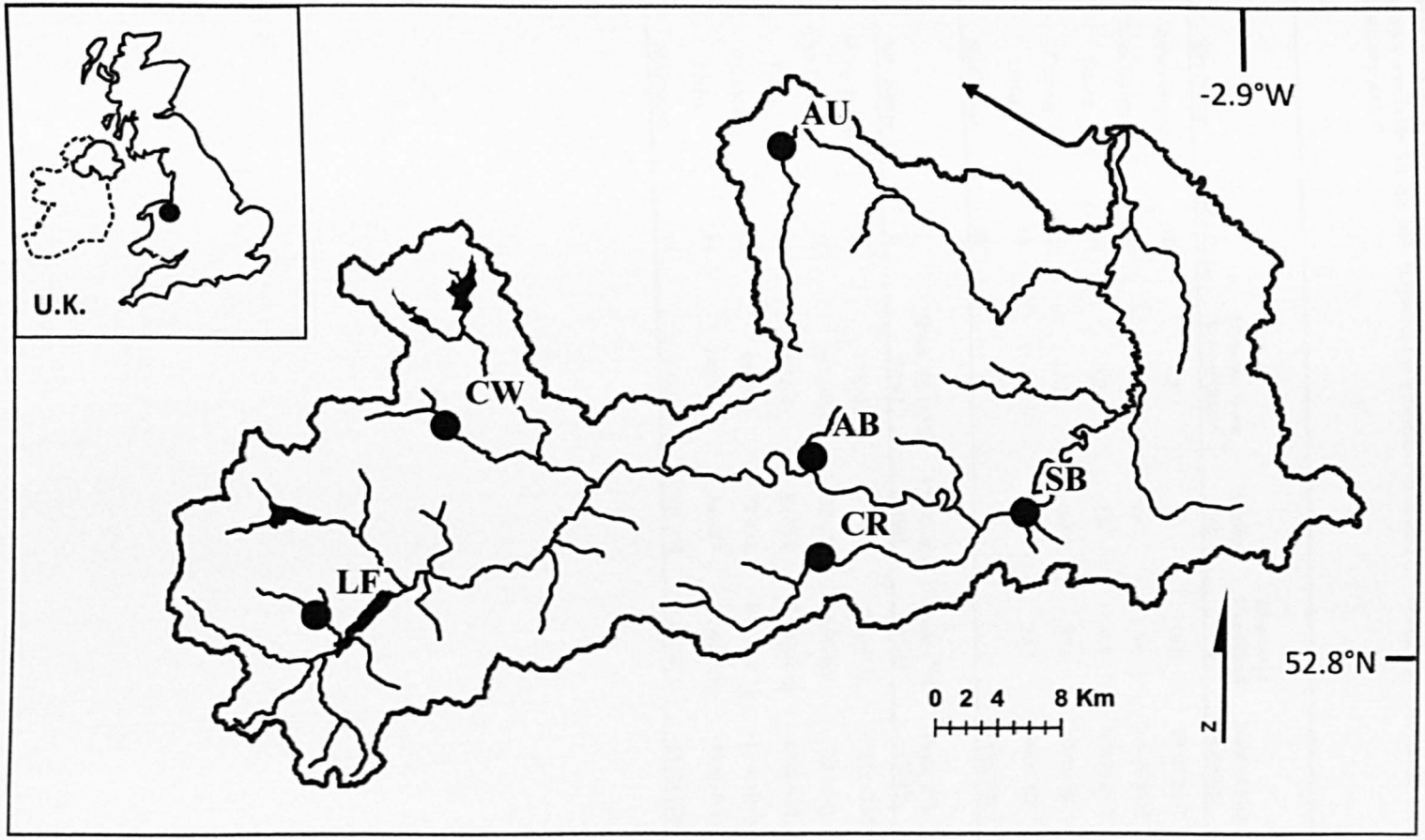

Figure 1. Location of the Dee catchment showing the catchment boundary and main river network (river discharge point into the Dee estuary is shown with a black arrow). 2007 electrofishing survey sites (black circles) and corresponding site codes (Table 1) are also shown.

Table 1. Electrofishing site locations [British National Grid Reference (NGR)], percentage of site-subcatchments composed of arable land and improved grassland, date of fish capture, fish sample sizes (n), fork lengths and average $\delta^{15}N$, $\delta^{13}C$ and δD values in scales of *Salmo trutta* from 6 sites in the Dee river catchment. No land use data were available for site SB. *Denotes the downstream distance between the start of the headwater source and each survey site.

Site Name	Site Code	Distance from Source (km)*	Arable (%)	Improved Grassland (%)	Date of Fish Capture	NGR
Abbey Brook	AB	4.8	0.3	18.9	10/08/2007	SJ206444
Alyn Upstream	AU	15.4	2.4	53	14/09/2007	SJ188653
Ceirw	CW	5.9	1.9	6.5	09/08/2007	SH1962465
Ceiriog	CR	14.2	0.4	38.6	10/08/2007	SJ211377
Llafer	LF	4.9	0	33.4	09/08/2007	SH1877338
Shellbrook	SB	2.9	-	-	13/09/2007	SJ348407

Site Name	n	Mean FL (±SE) (mm)	FL Range (mm)	Scale $\delta^{15}N$ (±SE)	Scale $\delta^{13}C$ (±SE)	Scale δD (±SE)
Abbey Brook	11	140(±5)	113-181	9.5(±0.2)	-22.9(±0.3)	-61.4(±1.9)
Alyn Upstream	16	187(±12)	132-258	10.9(±0.2)	-24(±0.2)	-58.4(±1.4)
Ceirw	20	157(±9)	83-250	13.4(±0.1)	-22.4(±0.1)	-58.4(±1.8)
Ceiriog	13	141(±8)	72-198	10.07(±0.1)	-19.4(±0.1)	-59.8(±2.13)
Llafer	15	148(±10)	86-257	11.8(±0.1)	-20.6(±0.1)	-59.4(±2.2)
Shellbrook	14	169(±9)	109-222	12.6(±0.2)	-23.7(±0.2)	-57.7(±1.0)

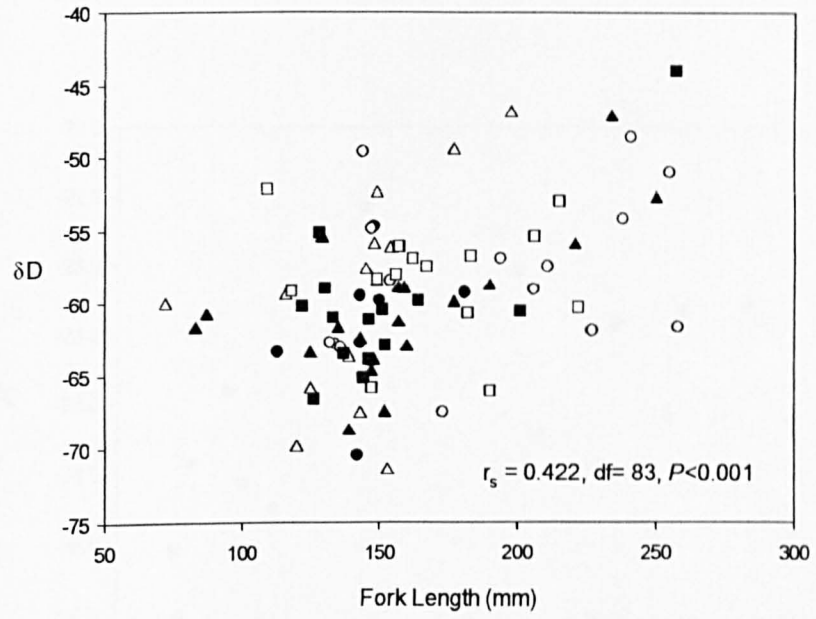

Figure 2. Scatter plot of *Salmo trutta* fork length and δD in scales at 6 sites in the Dee catchment (AB, closed circles; AU, open circles; CW, closed triangles; CR, open triangles; LF closed squares and SB, open squares). The correlation using Spearman's Rank Correlation Coefficient (r_s) between δD in scales (pooled across all sites) and *Salmo trutta* fork length is also shown.

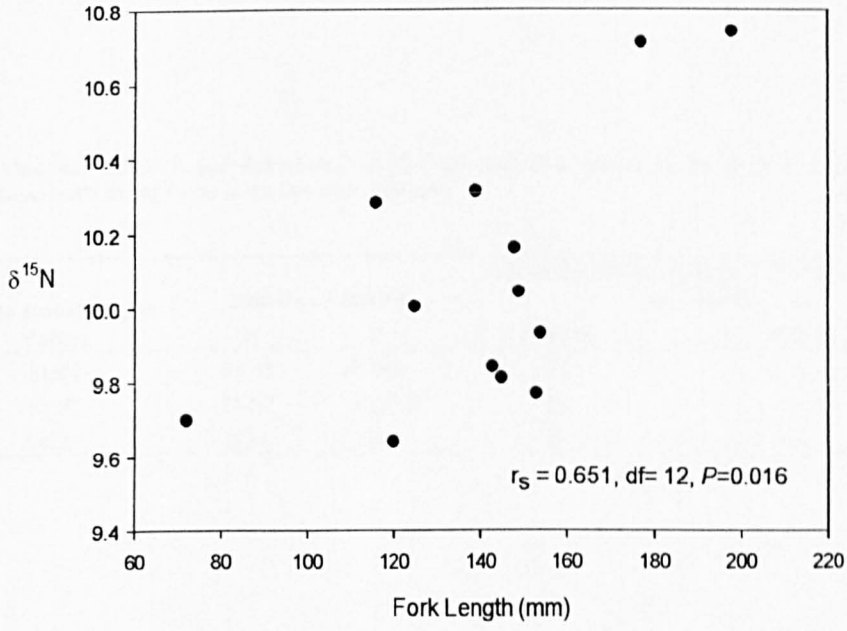

Figure 3. Scatter plot of $\delta^{15}N$ in scales of *Salmo trutta* (n=13) and fork length at site CR in the Dee catchment. The correlation using Spearman's Rank Correlation Coefficient (r_s) between $\delta^{15}N$ in scales and *Salmo trutta* fork length is also shown.

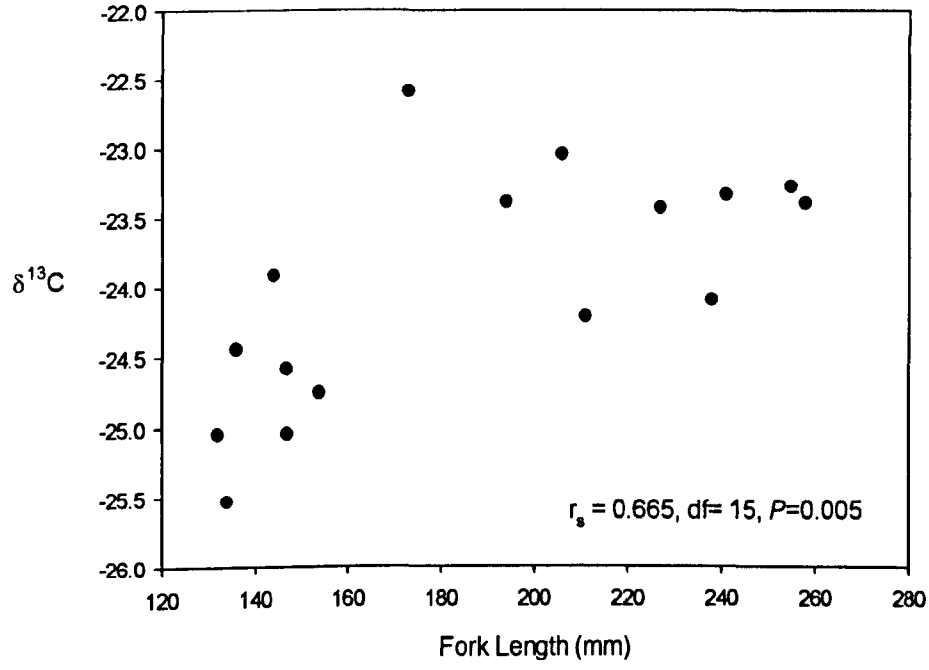

Figure 4. Scatter plot of $\delta^{13}C$ in scales of *Salmo trutta* (n=16) and fork length at site AU in the Dee catchment. The correlation using Spearman's Rank Correlation Coefficient (r_s) between $\delta^{13}C$ in scales and *Salmo trutta* fork length is also shown.

Table 2. One-way ANOVA and Scheffe's Post-Hoc pairwise comparison results for $\delta^{15}N$, $\delta^{13}C$ and δD in scales of *Salmo trutta* among 6 sites in the Dee river catchment.

Stable Isotope Delta Values	One-way ANOVA		No. of significant Scheffe's Post hoc pairs (out of 15)	
	F	*P*	*P*<0.05	*P*<0.001
δ15N	64.91	<0.001	12	10
δ13C	85.97	<0.001	13	12
δD	0.31	0.903	0	0

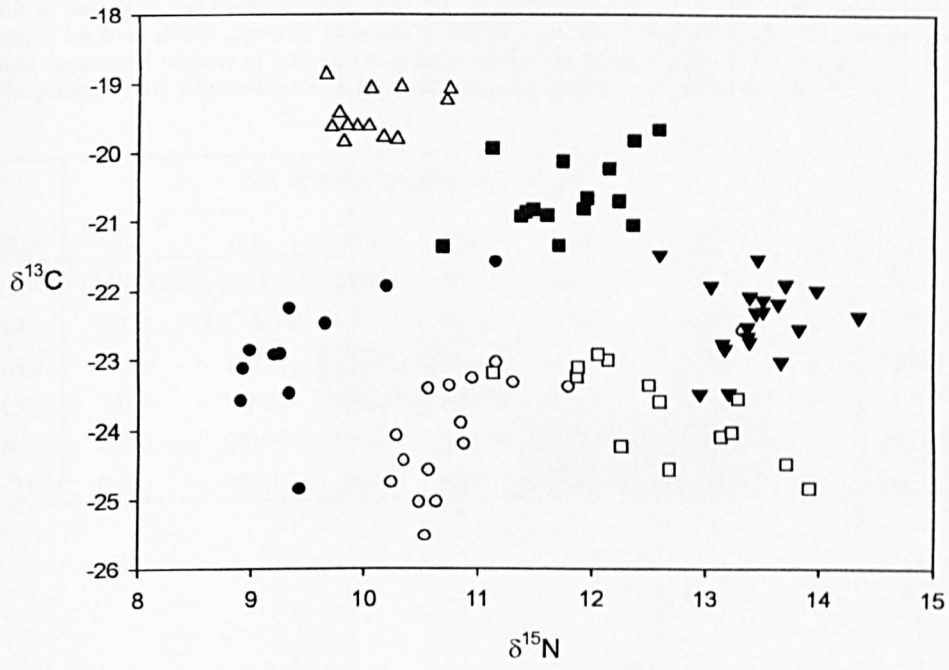

Figure 5. Scatter plot of $\delta^{15}N$ and $\delta^{13}C$ in scales of *Salmo trutta* from 6 sites in the Dee river catchment; CW (closed triangles), LF (closed squares), AB (closed circles), CR (open triangles), AU (open circles), SB (open squares).

Table 3. Summary of the classification to site of origin of *Salmo trutta* achieved using combinations of $\delta^{15}N$, $\delta^{13}C$ values and Ba, Mg, Mn and Sr concentrations in scales of *Salmo trutta* (n=80) from 6 sites in the Dee river catchment.

		LDFA	
Biogeochemical Tag	**MLR (%)**	**Original (%)**	**CV (%)**
$\delta^{15}N$	59	58	58
$\delta^{13}C$	64	69	63
$\delta^{15}N$, $\delta^{13}C$	99	93	93
Ba, Mg, Mn, Sr in scales	96	91	88
Ba, Mg, Mn, Sr in otoliths	95	93	89
$\delta^{15}N$, $\delta^{13}C$ & Ba, Mg, Mn, Sr in scales	100	98	96

Table 4. Cross-validation LDFA classification of fish to their site of origin based on $\delta^{15}N$, $\delta^{13}C$ in scales (in front of the slash) and concentrations of Sr, Ba, Mg and Mn in scales (between the slashes) and otoliths (behind the final slash). Correctly classified individuals are shown in bold. Light, medium and dark grey boxes correspond to pairs of sites in the upper, middle, and lower regions of the catchment respectively. *The percentage of individuals at each site which were correctly classified to their site of origin.

	No. of fish classified to each site							
Site	CW	LF	AB	CR	AU	SB	n	Correct (%)*
CW	13/14/14	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	14	93/100/100
LF	0/1/1	15/13/14	0/0/0	0/1/0	0/0/0	0/0/0	15	100/87/93
AB	0/0/0	1/0/0	9/7/11	0/4/0	1/0/0	0/0/0	11	82/63/100
CR	0/0/0	1/1/2	0/2/0	11/9/10	0/0/0	0/0/0	12	92/75/83
AU	0/0/0	0/0/0	0/0/0	0/0/0	13/13/14	1/1/0	14	93/93/100
SB	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	13/14/14	14	93/100/100

Chapter 6

Discussion

This thesis has provided a thorough assessment of whether biogeochemical tags might offer a useful research tool for improving our understanding of *Salmo trutta* population ecology in the U.K. While caution should be taken in extrapolating the results from this study to other species and geographical locations, the findings are broadly applicable to the use of biogeochemical tags in fisheries research in general, particularly regarding the application of biogeochemical tags in freshwater, which has received relatively little attention to date.

In review articles on the use of otolith microchemistry as a fisheries research tool, Campana (1999) and Gillanders (2005) outlined the criteria that need to be satisfied before fish structure chemistry can be used as natural tags of fish populations. While these authors defined these criteria in relation to the use of stable isotope ratios and element concentrations in otoliths as stock discrimination tools, they could be considered applicable to any natural tag used in fisheries research. The criteria from these two reviews have been outlined below.

Criteria 1. Fish residing in different locations must have distinct characteristics which can be used as natural tags of fish populations/groups.

Criteria 2. Fish samples (e.g. otoliths/scales) used to characterise the natural tag for each population/group should be representative of all fish in those populations/groups.

Criteria 3. The natural tags of all possible populations/groups of fish contributing to the mixed stock of fish of unknown origin must be characterised.

Criteria 4. The natural tag must remain stable for the interval between characterisation and the mixing of fish populations/groups. In particular:

4.1 Baseline natural tags for each population/group must remain temporally stable.

4.2 The natural tag once formed in the fish must remain stable (e.g. no significant post-depositional change in scale hydroxyapatite must occur).

Criteria adapted from Campana (1999) and Gillanders (2005)

In order to provide a rigorous assessment of the use of biogeochemical tags in the research of *Salmo trutta* populations in the U.K., the current research thesis has attempted to assess the degree to which biogeochemical tags of *Salmo trutta* satisfy the criteria outlined above. The degree to which these criteria have been met and the implications of this for the application of biogeochemical tags as fisheries research tools for *Salmo trutta* are discussed below.

Determining the catchment of origin of sea-run *Salmo trutta* (Chapter 2)

In order to examine whether the catchment of origin of *Salmo trutta* could be determined using scale microchemistry, the freshwater hydroxyapatite growth bands of scales of *Salmo trutta* from 12 catchments were examined. There were significant differences in element concentrations among the 12 catchments (Chapter 2; Table 3)(Criteria 1). Scales samples were collected from fish in main river channel sites and it is therefore assumed that the fish were representative of the range of *Salmo trutta* scale chemistries within each catchment (Criteria 2).

Unfortunately the differences in element concentrations in scales of *Salmo trutta* were insufficient to constitute a unique elemental signature for fish for each of the 12 catchments. Given that there was little evidence of regional patterning in scale chemistry (Chapter 2; Table 4), it is highly unlikely that element concentrations could reveal the freshwater origins of *Salmo trutta* to a catchment or regional level. This is particularly clear when considering that only 12 of the 80 or more major catchments which might be contributing *Salmo trutta* to the Irish and Celtic Seas, were included in the study (Criteria 3). In this study the *Salmo trutta* scale samples were collected from an age range of *Salmo trutta*, representing hydroxyapatite formed in freshwater over a number of years (Chapter 2; Table 1). This ensured that the accuracy with which fish could be classified to their catchment of origin, took into account possible temporal variability in baseline element signatures in each catchment (Criteria 4.1). There was some evidence of post-depositional change in element concentrations in the hydroxyapatite growth bands of *Salmo trutta* (Chapter 2; Table 7) (Criteria 4.2) which might have further compromised the degree to which catchment- or regional-specific signatures could be determined. It is possible that catchment-specific elemental signatures could be present in otoliths, given that they are considered metabolically inert (Campana & Thorrold, 2001) and therefore unlikely to suffer post-depositional change in composition (Criteria 4.2).

Determining the origin of *Salmo trutta* to sites within a catchment (Chapters 3, 4 and 5)

In order to examine whether the intra-catchment origin of *Salmo trutta* could be determined using fish-structure chemistry, element concentrations in scales and otoliths of *Salmo trutta* from sites within the Dee catchment were examined. Significant differences in element concentrations in the otoliths and scales of *Salmo trutta* were found (Chapter 3; Table 3, Chapter 4; Table 2) (Criteria 1) across a range of fish sizes (Chapter 3; Table 1, Chapter 4; Table 1) (Criteria 2) from sites in the catchment. Using the BGS stream water chemistry data to predict Sr and Mn concentrations in the scales of *Salmo trutta* at 792 sites in the Dee catchment provided a unique opportunity to characterise element signatures in scales for the entire Dee catchment (Chapter 4; Fig. 7) (Criteria 3). The

correlations between element concentrations in scales and in the 2007 stream water dataset and 1988-1994 stream water dataset suggest that the element signatures in the scales of *Salmo trutta* might be temporally stable on decadal timescales (Criteria 4.1). While there is evidence of post-depositional change in the element concentrations in the hydroxyapatite growth bands of *Salmo trutta* both Mn and Sr were highly correlated in scales and otoliths (Chapter 3; Figure 2). Given that otoliths are considered metabolically inert (Campana & Thorrold, 2001) and therefore unlikely to suffer post-depositional change in composition (Criteria 4.2), they might provide an alternative to scales. Unfortunately, while there appeared to be some regional patterning in scale chemistry (Chapter 4; Fig. 5 & 6), there are probably geographically isolated areas of the catchment which share similar scale chemistries (Chapter 4; Fig. 7 & 8).

The use of $\delta^{13}C$ and $\delta^{15}N$ in scales as non-lethally collectable biogeochemical tags of fish in the Dee catchment shows promise. Significant differences were found in $\delta^{13}C$ and $\delta^{15}N$ in scales from 6 sites in the Dee catchment (Chapter 5; Table 2) (Criteria 1). The temporal and spatial variability in $\delta^{13}C$ and $\delta^{15}N$ in the Dee catchment would need to be determined (Criteria 3 and 4) before the applicability of the technique as a *Salmo trutta* research tool could be confirmed.

The experimental design of the studies in this research thesis has taken into consideration the four criteria outlined by Campana (1999) and Gillanders (2005). Unfortunately the applicability of element concentrations in scales as a non-lethal tool for determining the catchment of origin of *Salmo trutta* in the U.K. and the applicability of element concentrations in scales and otoliths for determining the intra-catchment site of origin of the species is probably limited as both applications of the technique failed to satisfy one or more of the Criteria. However, the findings of the research have provided a useful contribution to the expanding literature on biogeochemical tags in fisheries research. Perhaps of particular significance are the methodological developments, such as highlighting the importance of determining how the baseline element signatures in fish calcified structures might vary at fine spatial scales. Expanding the suite of element concentrations used as biogeochemical tags provided a limited improvement in the discrimination of fish to site of origin (Chapter 3). While the

suitability of particular biogeochemical tags for determining fish origins is probably dependant on the species in question and the geographical location, this research thesis found that combining the stable isotope ratios and element concentrations in scales of *Salmo trutta* improved the accuracy with which the site of origin of fish could be determined within a catchment. Based on the findings of the research thesis, some suggestions for future research have been outlined below.

Recommendations for Future Research

Methods for determining baseline element signatures and/or stable isotope ratios in calcified structures of fish in stock discrimination studies usually involve sampling fish structure chemistry at a limited number of sites in the study region (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005; Clarke *et al.*, 2007) and therefore rarely account for all possible sources of fish contributing to the mixed stock (Criteria 3) and assumes that no inward fish migration occurs from beyond the study boundary. In these cases, the high degree of accuracy with which fish can be classified to their site of origin, is probably dependant on the number of sites included in the study and does not reflect the true accuracy with which the geographical origin of fish can be determined. While it is usually prohibitively expensive to determine baseline signatures at all locations inhabited by fish, future studies could adopt a hierarchical sampling design which examines fish structure chemistry at different spatial and temporal scales.

A broader suite of element concentrations in the hydroxyapatite of scales of *Salmo trutta* exhibited spatial variability among geographical locations in the Dee catchment when compared with element concentrations in otoliths. The findings of this study suggest that scales might offer a non-lethal biogeochemical tag of fish, comparable in performance to otoliths. However, future work needs to examine the degree of post-depositional change in scale hydroxyapatite. This would probably require aquaria-based studies in which the effect on scale chemistry of exposing fish to changing element concentrations in water and diet could be monitored.

It was beyond the scope of the current research thesis to examine the performance of stable isotope ratios, other than $\delta^{15}N$ and $\delta^{13}C$, as biogeochemical tags of fish. Dual $\delta^{15}N$ and $\delta^{13}C$ can be carried out relatively quickly and inexpensively (Harrington *et al.*, 1998). However, future studies could consider using $^{87}Sr:^{86}Sr$, $\delta^{34}S$ and $\delta^{18}O$ in fish structures as these stable isotope ratios are also emerging as powerful biogeochemical tags of fish (Weber *et al.*, 2002; Leakey *et al.*, 2008). Variability in $^{87}Sr:^{86}Sr$ among freshwater locations can be driven by variations in bedrock geology (Barnett-Johnson *et al.*, 2008). Metamorphic and granite rocks usually have high $^{87}Sr:^{86}Sr$ whereas streams draining carbonate rocks have lower values. $^{87}Sr:^{86}Sr$ can be incorporated into otoliths in direct proportion to their ratios in stream water (Kennedy *et al.*, 1997) and can exhibit relatively little temporal change (Kennedy *et al.*, 2000). $^{87}Sr:^{86}Sr$ might offer a particularly useful biogeochemical tag of fish in the U.K. For instance, the heterogeneous geology in the Dee catchment [ranging from Ordovician and Silurian to Carboniferous and Triassic (Chapter 4)] might generate regional differences in $^{87}Sr:^{86}Sr$ in stream water (and therefore otolith aragonite and scale hydroxyapatite) over relatively small spatial areas within the catchment. $^{87}Sr:^{86}Sr$ in otoliths have been successful in revealing movement patterns of fish in large watersheds, such as the Ganges (>163,000km^2)(Milton & Chenery, 2005). The Dee catchment (~1800km^2), is much smaller in size with many contrasting lithologies (Chapter 4), which might provide an opportunity to examine fish origins/movements over small spatial scales using $^{87}Sr:^{86}Sr$ in fish calcified structures.

In order to enhance the discrimination of fish to their geographical location of origin, consideration should also be given to combining biogeochemical tags with other 'natural' tags of fish, such as morphometric and genetic differences among fish populations (reviewed in Begg & Waldman, 1999). The relative suitability of a given tagging method will probably depend on the nature of the research question, the geographical location of the study and the species concerned. For instance, in situations where mixed stocks of fish originate from different geographical locations but which interbreed to a degree, a combination of genetic and calcified structure chemistry might offer the best discrimination of fish populations (Bronte *et al.*, 1996; Feyrer *et al.*, 2007). For instance, Feyrer and co-authors (2007) found that genetic analysis distinguished

two reproductively distinct populations of the splittail (*Pogonichthys macrolepidotus*) from natal rivers discharging into San Francisco Bay estuary in California and otolith element concentration data distinguished a further four populations originating from the four main rivers discharging into the Bay (Feyrer *et al.*, 2007). Brenkman & Corbett (2007) found that a combination of natural tags (otolith chemistry) and artificial tags (radio-telemetry) provided complementary data on the life history of anadromous bull trout (*Salvelinus confluentus*).

While the application of fish structure chemistry has generated particular interest as a stock discrimination tool for wild populations of fish, a number of other applications have also been identified. A recent study provided a comprehensive evaluation of the use of element concentrations in scales to distinguish between farmed and wild Atlantic salmon (*Salmo salar*) (Adey *et al.*, 2009). The high degree of accuracy with which the two groups could be distinguished suggests that element concentrations in calcified structures of fish might have significant application for distinguishing between wild *Salmo trutta* and farm escapees of the species.

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Appendix A

Techniques for determining fish calcified structure chemistry

Element Concentrations

In recent years Inductively Coupled Plasma Mass Spectrometry (ICP-MS) has been described as the instrument of choice for the determination of multi-element concentrations in fish calcified structures (Campana, 1999; Gillanders, 2001; Gillanders, 2005).

The fundamental principle of ICP-MS is the generation of positively charged ions. Sample material is introduced as an aerosol to a high temperature plasma (usually argon) where the sample is atomised and ionised (Thomas, 2001). Ions are formed by collision of atoms (originating from the sample) with the energetic argon electrons in the plasma so that the outer electron is removed, thus forming a positively charged ion (Thomas, 2001). After emerging from the plasma, the ions are directed towards the mass spectrometer. The ions will have different kinetic energies based on their mass-to-charge ratio, allowing the ionic composition of the sample material to be determined (Thomas, 2001). A suite of elements can be measured simultaneously at levels in the parts per billion (ppb) range (Jones & Chen, 2003).

ICP-MS facilities were commercially available from 1984 onwards and initially relied on a solution-based sample introduction (Jones & Chen, 2003). While small areas of solid samples could be isolated using micro-milling and then dissolved in preparation for a solution-based sample introduction, the need to target small spatial areas in solid samples (particularly in geological research) led to the development of Laser Ablation ICP-MS (LA-ICP-MS). Coupling a laser to the ICP-MS allowed small spatial areas within a sample to be targeted for analysis (e.g. >4μm diameter spot ablation) (Jones & Chen, 2003). In terms of fish-chemistry, this allowed element concentrations in particular growth rings of fish calcified structures to be sampled relatively easily (Fowler *et al.*, 1995).

There are tradeoffs between the use of solution-based ICP-MS and LA-ICP-MS. In contrast to solution-based ICP-MS, the laser generates particle sizes of the sample which are less optimal for ionization in the plasma (Jones & Chen,

2003). However, the water used in solutions of dissolved samples (ICP-MS) has been found to be an important source of ions in the formation of polyatomic species (e.g. ^{56}Fe suffers interference from $^{40}Ar^{16}O$) (Jarvis, 1997). Although detection of trace elements using LA-ICP-MS can be as low as a few ng/g (Mason & Mank, 2001), the limits of detection (LOD) are generally higher when compared with solution-based ICP-MS. Another advantage of solution-based analysis is that the analytical accuracy and precision is generally superior when compared with that achieved using LA-ICP-MS (Campana, 1999; Jones & Chen, 2003). Precision using solution-based ICP-MS is typically <1% relative standard deviation (RSD) of standards compared with a precision using LA-ICP-MS of typically <10% RSD (Jones & Chen, 2003). Unfortunately given that solution-based analysis requires sample material to be dissolved, the opportunity to analyse small areas of a sample is limited. Bagenal *et al.* (1973) used a scalpel to isolate the *Salmo trutta* scale hydroxyapatite which was formed in freshwater, from the hydroxyapatite formed during marine residency. For the present study, LA-ICP-MS was used rather than solution-based ICP-MS because the sample preparation time was relatively rapid and the laser allowed seasonal growth bands in *Salmo trutta* calcified structures to be isolated for analysis, using a small laser spot ablation (122µm in diameter).

Because the weight of sample material removed by the laser can vary, the technique relies on there being an element in the sample of constant concentration that can be used as an internal standard, against which the concentrations of other elements can be measured. Essentially the element concentration data are element:internal standard ratios. Providing that the concentration of the internal standard in the sample material is known (per unit weight of sample), the concentrations of other analytes can be estimated in relation to this. In scale hydroxyapatite and otolith aragonite, the concentration of Ca typically varies by <1% (Clarke *et al.*, 2007) and is generally used as an internal standard (Wells *et al.*, 2003; Muhlfeld *et al.*, 2005).

Stable Isotope Ratios

Natural variability in stable isotope ratios has proven to be a useful ecological tool for studying food-web relationships (Gannes *et al.*, 1997) and for providing

biogeochemical tags of individuals/populations (reviewed in Hobson, 1999). A number of instruments have been used to determine stable isotope ratios in fish calcified structures. Using quadropole ICP-MS in isolation can lack sufficient precision for determining stable isotope ratios (Outridge *et al.*, 2002) but multi-collector ICP-MS (MC-ICP-MS) (Outridge *et al.*, 2002), has been useful for determining stable isotope ratios in fish calcified structures. Another instrument which has been used is Thermal Ionisation Mass Spectrometry (TIMS).

Isotope ratios are often reported as the ratio of the heavier isotope to the lighter isotope and are expressed as delta (δ) notation, which is defined as parts per thousand (‰) deviations from international standards.

Heavy atoms form more stable, stronger bonds because they vibrate more slowly than lighter atoms (Sulzman, 2007). This can lead to 'fractionation' in which the isotopic ratios in the source and product of a chemical transformation can differ (Sulzman, 2007). The fractionation process can lead to an 'enrichment' (increase in the ratio of the heavier isotope to the lighter isotope) or 'depletion' (decrease in the ratio of the heavier isotope to the lighter isotope). Stable isotope ratios assimilated by organisms can 'fractionate' so that the heavier isotope of an element accumulates in tissue more readily than the lighter isotope. For instance, $\delta^{15}N$ can be enriched from prey to predator (DeNiro & Epstein, 1981). Some biological processes (e.g. photosynthesis) can 'avoid' the heavier isotope and this is usually termed 'discrimination' (Sulzman, 2007). $\delta^{13}C$ and $\delta^{15}N$ are probably the most commonly measured isotope ratios in ecological studies and can be determined relatively inexpensively using dual measurement on the same sample.

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Appendix B

Factors influencing the incorporation of elements into scale hydroxyapatite and otolith aragonite

Changes in growth rates, ambient temperature and salinity are known to influence the incorporation of elements in calcified structures of fish (e.g. otoliths; Fowler *et al.*, 1995a; Fowler *et al.*, 1995b). The concentrations of elements in water/diet and physiological regulation of elements are also all likely to influence the availability/incorporation of elements into blood plasma and endolymph fluid (Payan *et al.*, 1997) and therefore the availability of elements at the site of scale and otolith structure formation. Fowler *et al.* (1995a & 1995b) have speculated that when the calcification rate varies, the 'potential' deposition rates of the various ions could also vary.

There is evidence that element concentrations in calcified structures can be influenced by differences between individual fish of the same species, such as differences in fish size (Belanger *et al.*, 1987; Brophy *et al.*, 2003). Generally speaking examining the effects of fish size on element concentrations has not been the focus of the studies in which it has been reported but it has been taken into consideration in studies examining differences in element concentrations in fish structures for stock discrimination purposes. Potential effects of fish size are explored partly because they pose a confounding factor if comparisons are being made between the chemistry of scale/otoliths of fish between sites where the size of fish among sites in the study varies. Some elements have been found to either increase or decrease in concentration with otolith size. Given that generally speaking otolith size is highly correlated with fish size/age, it is difficult to elucidate the degree to which such a relationship is driven by changes in the depositional rate of otoliths, or the dietary or physiological changes associated with increased fish size/age (Swearer *et al.*, 2003). Swearer commented that element concentrations in otoliths tended to decline with age (Swearer *et al.*, 2003). Belanger *et al.*, 1987 found that gender and size were related to element concentrations in scales of striped bass (*Morone saxatilis*). Most elements were negatively correlated with age and size (Belanger *et al.*, 1987). Brophy and co-

authors (2003) also found that the otolith diameter of juvenile herring (*Clupea harengus*) was inversely related to concentrations of Mg, Zn and Pb.

Variability in structure chemistry has been found between species inhabiting the same geographical area. In a study by Swearer and co-authors (2003) which investigated inter-species variability in element concentrations in otoliths, significant differences were found in the concentration of elements (particularly Sr and Ba) in otoliths of five estuarine species. Species which were closely related had more similar element concentrations in otoliths compared with species which were more distantly related (Swearer *et al.*, 2003). This was attributed to varying exposure and/or differences to the element in question and/or differences in the elemental incorporation or to differences in diet and physiology (Swearer *et al.*, 2003). Piscivory may lead to bioaccumulation of elements which might also explain differences between species (Swearer *et al.*, 2003). Given that it was unclear whether the individuals were from the same year class and/or whether intra-species geographical habitat use was the same, it is difficult to attribute the differences in otolith chemistry to, for example, differences in physiology or diet (Swearer *et al.*, 2003). Gillanders & Kingsford (2003) found element concentrations differed among the otoliths of three species of sparids inhabiting the same geographical area. These differences might have been attributable to differences in micro-habitat use, among other factors (Gillanders & Kingsford, 2003). Hamer and Jenkins (2007) compared element concentrations in two demersal marine fish species [snapper (*Pagrus auratus*) and sand flathead (*Platycephalus bassensis*)]. LA-ICP-MS was used to analyse the element concentrations of sectioned otoliths from the two species. The most recently formed region of the otoliths was targeted for analysis, corresponding to otolith material most likely to have been formed at the site of capture (Hamer & Jenkins, 2007). While incorporation rates of elements varied, concentrations of Ba were correlated between the two species (Hamer & Jenkins, 2007). In a laboratory-based study, Geffen *et al.*, (1998) found that the levels of Hg and Pb in the otoliths of three species varied despite identical rearing conditions (Geffen *et al.*, 1998).

Salinity and temperature have been found to affect both stable isotope ratios and the concentrations of some elements in fish structures (Fowler *et al.*, 1995a). For instance, $\delta^{18}O$ (Høie *et al.*, 2004) and Sr (Radtke, 1989) in otoliths

can vary depending on ambient temperature experienced by fish. It has been suggested that changes in temperature might induce biological effects that influence element concentrations in otoliths (Elsdon & Gillanders, 2002). Temperature changes may also affect the proteinaceous compounds surrounding the otolith, causing a change in the otolith crystal morphology from hydroxyapatite to vaterite which might influence the uptake of elements in otoliths (Kalish, 1989; Brown & Severin, 1999; Elsdon & Gillanders, 2002). Most studies examining the influence of salinity and temperature on fish calcified structure chemistry have been carried out in laboratory conditions in order that salinity and temperature can be manipulated and other variables, such as element concentrations in ambient water, can be controlled. The findings are sometimes inconsistent (Campana *et al.*, 1999). For instance, the effect of temperature on element concentrations is not always present (Wells *et al.*, 2000). Wells *et al.*, (2000) conducted a laboratory-based study on scales of juvenile spot (*Leiostomus xanthurus*) and the incorporation of elements in tank water, into the species scales and otoliths. Sr:Ca, Cd:Ca, Ba:Ca in scales reflected differences in ambient concentrations of the elements in water and were not influenced by temperature. Other studies have found that the effects varied depending on the element and can probably also depend on the combined interactions between factors (e.g. the effect of temperature and salinity combined) (Fowler *et al.*, 1995a; Fowler *et al.*, 1995b).

Fowler and co-authors (1995a and 1995b) suggested that factors controlling otolith chemistry are complex and may interact. The authors conducted studies on the effects of temperature and salinity on the composition of 23 elements in otoliths (Fowler *et al.*, 1995a; Fowler *et al.*, 1995b). Experiments involved three treatment regimes (salinity, temperature and salinity x temperature). When considered independently, both salinity and temperature had an effect of otolith chemistry. Temperature had the greatest influence on element concentrations of the elements. Mg, Mn, Cu, and Zn decreased in concentration in otoliths with an increase in temperature while Fe and Sr increased with increasing temperature. It should be noted that otolith growth also increased with temperature and this may have been a confounding factor and the authors pointed out that this might lend support to the theory that physiological factors such growth rates can effect otolith composition (see paragraph above)

(Fowler *et al.*, 1995a; Fowler *et al.*, 1995b). Elson and Gillanders (2002) also investigated the effect of salinity and temperature as single factors and as combined factors. Both temperature on its own and temperature and salinity combined had significant effects on Sr:Ca, Ba:Ca ratios and concentrations of $\delta^{13}C$ and $\delta^{18}O$. Salinity on its own did not influence element ratios but did influence $\delta^{13}C$ and $\delta^{18}O$. Mg:Ca and Mn:Ca showed little or no effect of temperature or salinity.

It appears that there are a number of factors which can affect the concentration of elements in fish calcified structures. These should be taken into consideration when using scale and otoliths as biogeochemical tags. The applicability of fish structure chemistry as a stock discrimination tool usually depends on there being detectable and temporally stable differences in element concentrations in fish structures among geographical locations, irrespective of whether these differences are due to variations in temperature, diet or fish physiology. It should be noted that the effect of temperature and salinity can be minimal in comparison to the effect of ambient concentrations of elements in water, for example, Elsdon & Gillanders (2005) found that the effect of salinity and temperature on Ba:Ca in otoliths was minimal compared with the effect of ambient Ba:Ca in water.

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Appendix C

LA-ICP-MS transect data for sea-run *Salmo trutta* (sea trout) scales

The following appendix contains the LA-ICP-MS data for sea-run *Salmo trutta* (sea trout) scales, showing differences in concentrations of Sr:Ca, Ba:Ca and Mn:Ca in scale hydroxyapatite formed during juvenile growth in freshwater and subsequent growth in the marine environment.

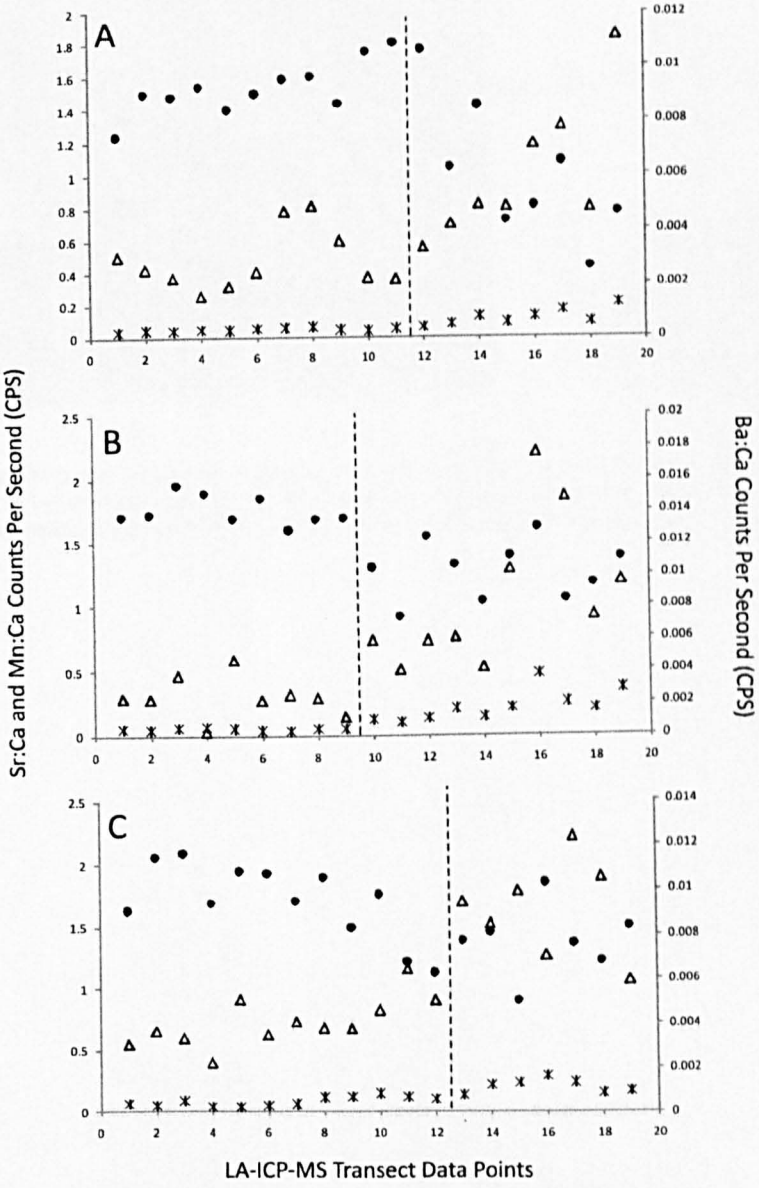


Figure 1. Sr:Ca (circles), Mn:Ca (stars) and Ba:Ca (triangles) in the hydroxyapatite of sea-run *Salmo trutta* (sea trout) scales determined by LA-ICP-MS transect analyses. Plate A. and B. correspond to data for one scale from each of two sea trout from the Border Esk catchment. Plate C. corresponds to data for one scale from a sea trout from the Taff catchment. The LA-ICP-MS Transect Data Points (n=20) correspond to a line transect starting in the hydroxypatite deposited during growth of the fish in the marine environment (from Data Points 0) which continues through to growth of the fish in the freshwater environment (finishing at Data Point 20)(Fig. 2). The dashed line is the 'smolt line' (corresponding to the transition of the fish between freshwater and the marine environment). The ratios are calculated as the Counts Per Second (CPS) of the isotopes ^{88}Sr, ^{55}Mn and ^{138}Ba to ^{48}Ca.

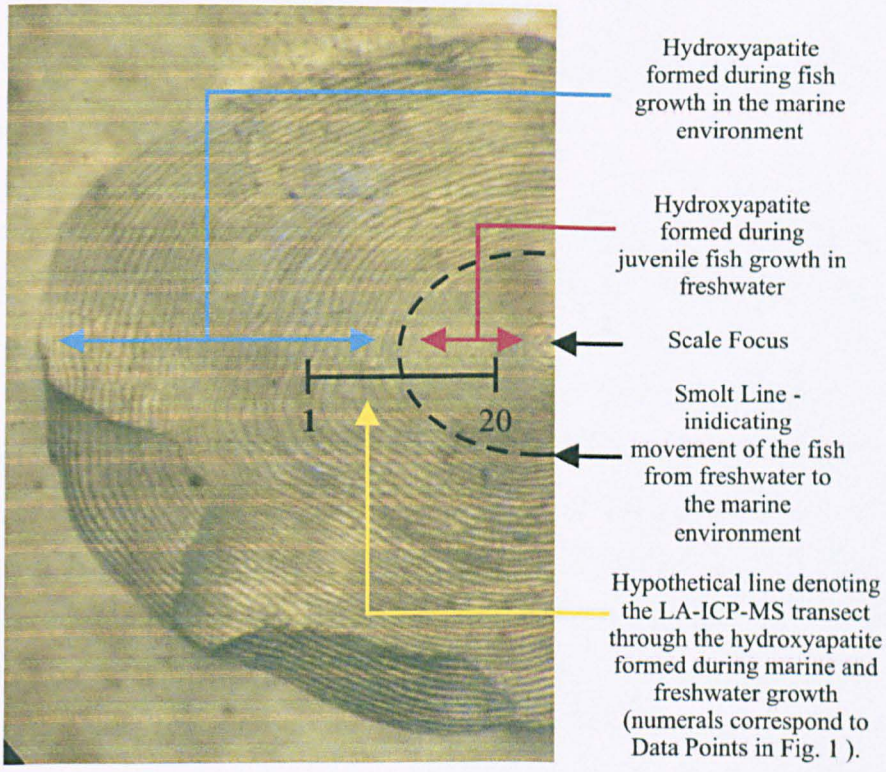


Figure 2. Image of a sea trout scale annotated with scale characteristics which were determined through scale reading. A hypothetical line denotes the start and end points of the LA-ICP-MS transects (Fig. 1.) which was conducted through the hydroxyapatite of the scale formed during marine and freshwater growth.

Appendix D

LA-ICP-MS data for *Salmo trutta* smolt scales collected from four catchments on the East coast of Ireland in 1993

The following appendix contains details of the LA-ICP-MS data for *Salmo trutta* smolt scales collected from four rivers (Nanny, Dargle, Slaney and Colligan) on the East coast of Ireland. Scale samples were removed from fish in 1993 and were kindly donated by the Central Fisheries Board, Ireland. A ban on sea trout fishing in Ireland prohibited the collection of sea trout scale samples from Irish rivers in 2007 and so the *smolt* scale samples from 1993 were originally intended to be used as a substitute for *sea trout* scale samples from Ireland as part of the study in Chapter 2. However, due to the potential for post-depositional change in scale hydroxyapatite (Chapter 2) and the fact that the Irish scale samples were collected from smolts several years earlier than those collected from sea trout for catchments in the U.K. (Chapter 2), the element concentration data for smolt scales from the Irish rivers have been analysed separately and presented here. The scale sample preparation procedures and the analytical methods are the same as those described for the sea trout scales samples in Chapter 2.

While there were significant differences in concentrations of Zn, Sr and Ba in scales of *Salmo trutta* collected from different catchments in Ireland (Table 1), only 48% of fish could be classified to their catchment of origin [using cross-validation Linear Discriminant Function Analysis (LDFA)], based on the concentrations of these elements in their scales. At a catchment level, the classification accuracy ranged between 38% (for the Slaney) and 55% (for the Colligan) (Table 2). There appears to be relatively little separation of fish among catchments based on element concentrations in their scales (Fig. 2). It is therefore unlikely that element concentrations in scales will offer a useful biogeochemical tag for distinguishing the freshwater origins of *Salmo trutta* originating from the East coast of Ireland.

N.B. Fish fork length data are missing for the majority of smolts included in the study and so no adjustment could be made for the effect of fish size on element

concentrations. However, the smolts were of a similar age (mean age = 2 years) and so are unlikely to vary considerably in size among rivers. Therefore any effect of fish size on element concentrations is unlikely to influence the Linear Discriminant Function Analysis (LDFA) and Multinomial Logistic Regression (MLR) results (Fig. 2 and Table 2).

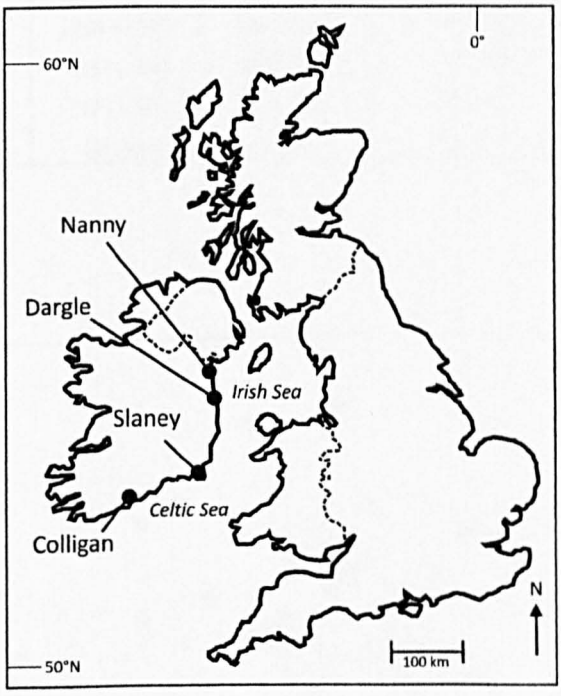


Figure 1. Map of U.K. and Ireland showing the river catchments from which *Salmo trutta* smolt scale samples were collected. Sample sizes in each catchment were as follows: Nanny (n=10), Dargle (n=14), Slaney (n=8) and Colligan (n=22). Fish were caught in 1993.

Table 1. Mean element concentrations in the freshwater growth bands of scales of *Salmo trutta* smolts from 4 catchment on the East coast of Ireland. Mean smolt age was 2 years. One-way ANOVA and Kruskal-Wallis were used to determine if there were significant differences in element concentrations in *Salmo trutta* smolt scales collected from different catchments. The number of significant pairwise comparisons (using Scheffe's post-hoc test) are shown.

Element	Mean element concentrations in the freshwater growth bands of *Salmo trutta* smolt scales from 4 catchments on the East coast of the U.K. (µg/g) (±1SE) (n = number of fish in each catchment)				ANOVA		Number of significant post-hoc pairs (P<0.05)
	Nanny (n=10)	Dargle (n=14)	Slaney (n=8)	Colligan (n=22)	$F_{3,53}$	P	
Li	-2.5 (±2.7)	0.6 (±0.6)	0.2 (±0.3)	3.7 (±1.7)	1.91	0.14	0
Mg	7416 (±1711)	7137 (±1019)	6895 (±1467)	8139 (±616)	0.93	0.43	0
Mn	763 (±230)	693 (±131)	639 (±243)	617 (±114)	0.28	0.84	0
Cu	83 (±73)	11 (±4)	4 (±1)	21 (±9)	0.14	0.93	0
Zn	1027 (±263)	1984 (±293)	905 (±212)	899 (±102)	5.47	0.002	3
Sr	1200 (±111)	1357 (±86)	982 (±114)	1392 (±59)	4.50	0.007	2
Ba	74 (±8)	97 (±28)	49 (±10)	42 (±5)	3.58	0.02	0
Pb	10 (±4)	10 (±3)	3 (±1)	5 (±2)	0.78	0.51	0

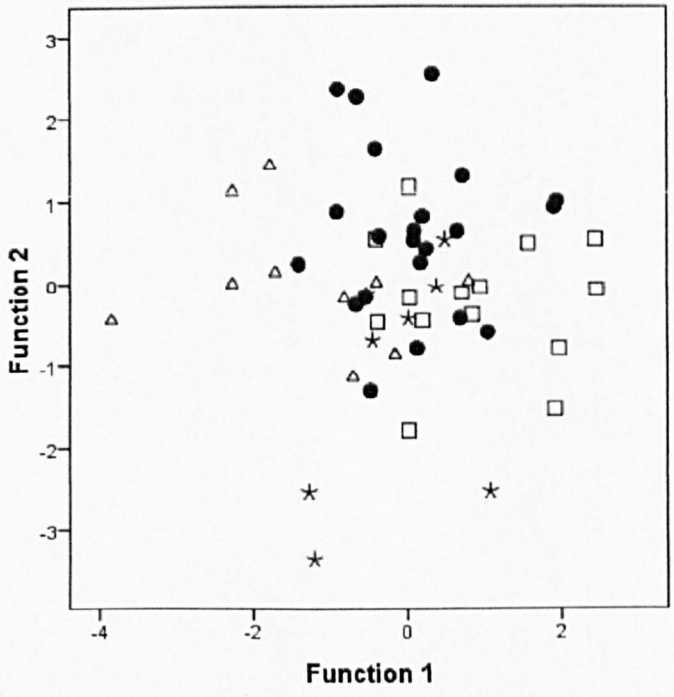


Figure 2. Canonical variate scores plot of the first two discriminant functions in the LDFA based on concentrations of Li, Mg, Mn, Cu, Zn, Sr, Ba and Pb in *Salmo trutta* smolt scales samples from the Nanny (triangles), Dargle (squares), Slaney (stars) and Colligan (circles) rivers in Ireland (Fig. 1).

Table 2. Cross validation (CV) Linear Discriminant Function Analysis (LDFA) classification of *Salmo trutta* smolts to their catchment of natal origin based on concentrations of Li, Mg, Mn, Cu, Zn, Sr, Ba and Pb in scales. Correctly classified individuals are shown in dark grey boxes and highlighted in bold. The light grey boxes correspond to study catchments neighbouring the natal catchments of origin. *Denotes the percentage of individuals correctly classified to their catchment of origin using cross-validation LDFA. The LDFA resulted in a 61% 'original' and a 48% CV classification. The MLR classification accuracy (equivalent to the original LDFA classification) was 69%.

Catchment	No. of *Salmo trutta* smolts classified to their catchment of natal origin based on element concentrations in the freshwater growth bands of scales				n	% Classified to natal catchment*
	Nanny	Dargle	Slaney	Colligan		
Nanny	**4**	2	2	2	10	40
Dargle	2	**7**	3	2	14	50
Slaney	1	1	**3**	3	8	38
Colligan	1	6	3	**12**	22	55

Appendix E

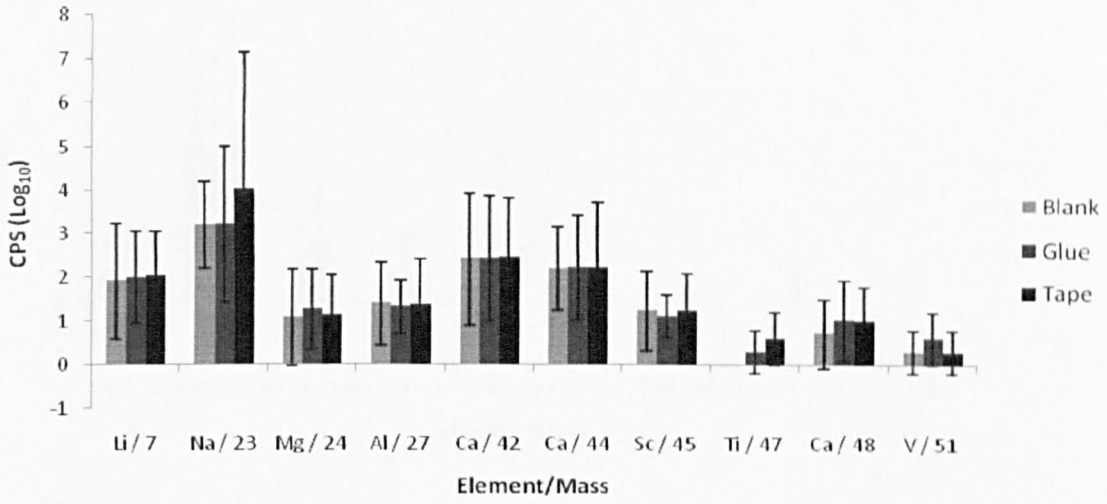
LA-ICP-MS analysis of glue and double sided tape

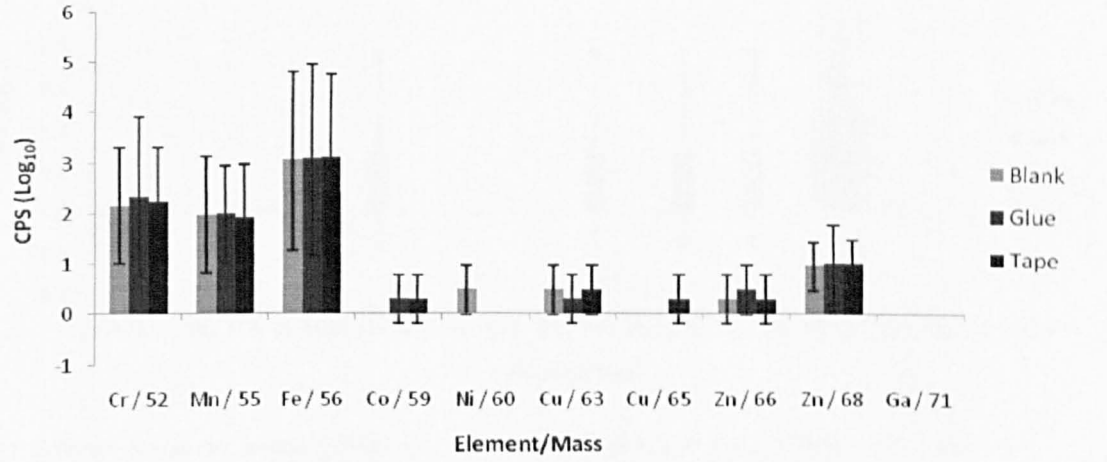
The glue (Araldite) and double-sided tape (No More Nails Tape) used to mount otoliths and scales respectively, in preparation for LA-ICP-MS analysis, were analysed on the NERC LA-ICP-MS facility (Kingston University) which comprises a VG Elemental PlasmaQuad 2+ STE coupled to a Cetac LSX-100 (wavelength 266nm) laser.

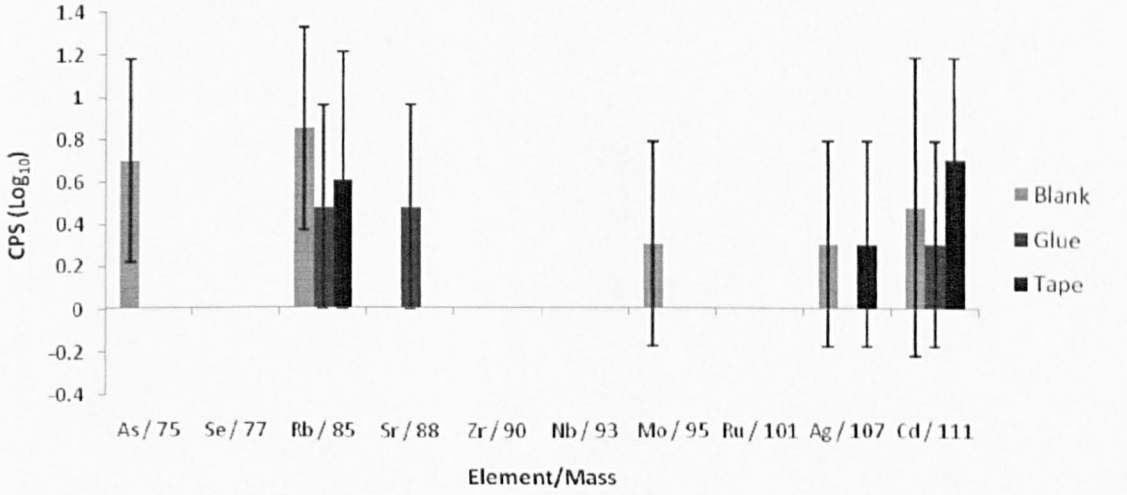
The counts per second (CPS) of each isotope in each of the materials is shown in Table 1 and Figure 1 (overleaf).

Table 1. Counts per second (CPS) of each isotope in the gas blanks (n=3), glue (Araldite) (n=3) and tape (No More Nails Tape) (n=3), as determined by LA-ICP-MS (n=number of measurements). Data for the glue and tape are not blank corrected.

	Counts Per Second (CPS)								
Element/Mass	Blank 1	Blank 2	Blank 3	Glue 1	Glue 2	Glue 3	Tape 1	Tape 2	Tape 3
Li / 7	100	60	77	93	110	93	110	113	97
Na / 23	1607	1593	1590	1714	1644	1764	10121	11285	12562
Mg / 24	0	10	23	10	23	20	7	20	13
Al / 27	20	20	33	17	23	20	33	13	23
Ca / 42	233	287	290	267	263	310	270	313	293
Ca / 44	153	163	170	187	163	160	147	203	157
Sc / 45	17	10	23	13	13	10	20	20	10
Ti / 47	0	0	0	0	3	0	3	7	0
Ca / 48	0	3	10	3	10	17	13	3	10
V / 51	3	0	0	0	3	7	0	0	3
Cr / 52	143	130	157	180	250	227	177	173	157
Mn / 55	107	80	97	90	97	107	73	80	93
Fe / 56	1153	1040	1113	1277	1167	1127	1167	1243	1253
Co / 59	0	0	0	3	0	0	3	0	0
Ni / 60	0	3	3	0	0	0	0	0	0
Cu / 63	3	0	3	3	0	0	3	0	3
Cu / 65	0	0	0	0	0	0	3	0	0
Zn / 66	0	0	3	3	3	0	3	0	0
Zn / 68	7	10	7	3	13	10	10	7	10
Ga / 71	0	0	0	0	0	0	0	0	0
As / 75	7	3	3	0	0	0	0	0	0
Se / 77	0	0	0	0	0	0	0	0	0
Rb / 85	7	3	7	3	0	3	0	3	7
Sr / 88	0	0	0	3	0	3	0	0	0
Zr / 90	0	0	0	0	0	0	0	0	0
Nb / 93	0	0	0	0	0	0	0	0	0
Mo / 95	3	0	0	0	0	0	0	0	0
Ru / 101	0	0	0	0	0	0	0	0	0
Ag / 107	0	3	0	0	0	0	0	0	3
Cd / 111	0	0	7	0	3	0	7	3	3
In / 115	0	0	3	3	0	0	0	0	0
Sn / 118	37	40	37	33	23	37	43	27	57
Cs / 133	0	0	0	3	0	0	0	3	7
Ba / 137	0	0	0	0	0	0	0	0	3
La / 139	0	0	0	0	0	0	0	0	0
Ce / 140	0	0	0	0	0	0	3	0	0
Pr / 141	0	0	0	0	0	0	0	0	0
Nd / 146	0	0	0	0	0	0	0	0	0
Sm / 147	0	0	3	0	0	0	0	0	0
Eu / 151	0	0	0	0	0	0	0	0	0
Gd / 157	0	0	0	0	0	0	0	0	0
Tb / 159	0	0	0	0	0	0	0	0	0
Dy / 163	0	0	0	0	0	0	0	0	3
Ho / 165	3	0	0	0	0	0	0	0	0
Er / 166	0	0	0	0	0	0	0	0	0
Tm / 169	0	0	0	0	0	0	0	3	0
Yb / 172	0	0	0	0	0	0	0	3	0
Lu / 175	0	0	0	3	0	0	0	0	0
Pb / 208	3	3	0	7	3	7	0	0	7
Th / 232	0	0	0	0	0	0	0	0	0







(Figure 1 and caption are continued overleaf)

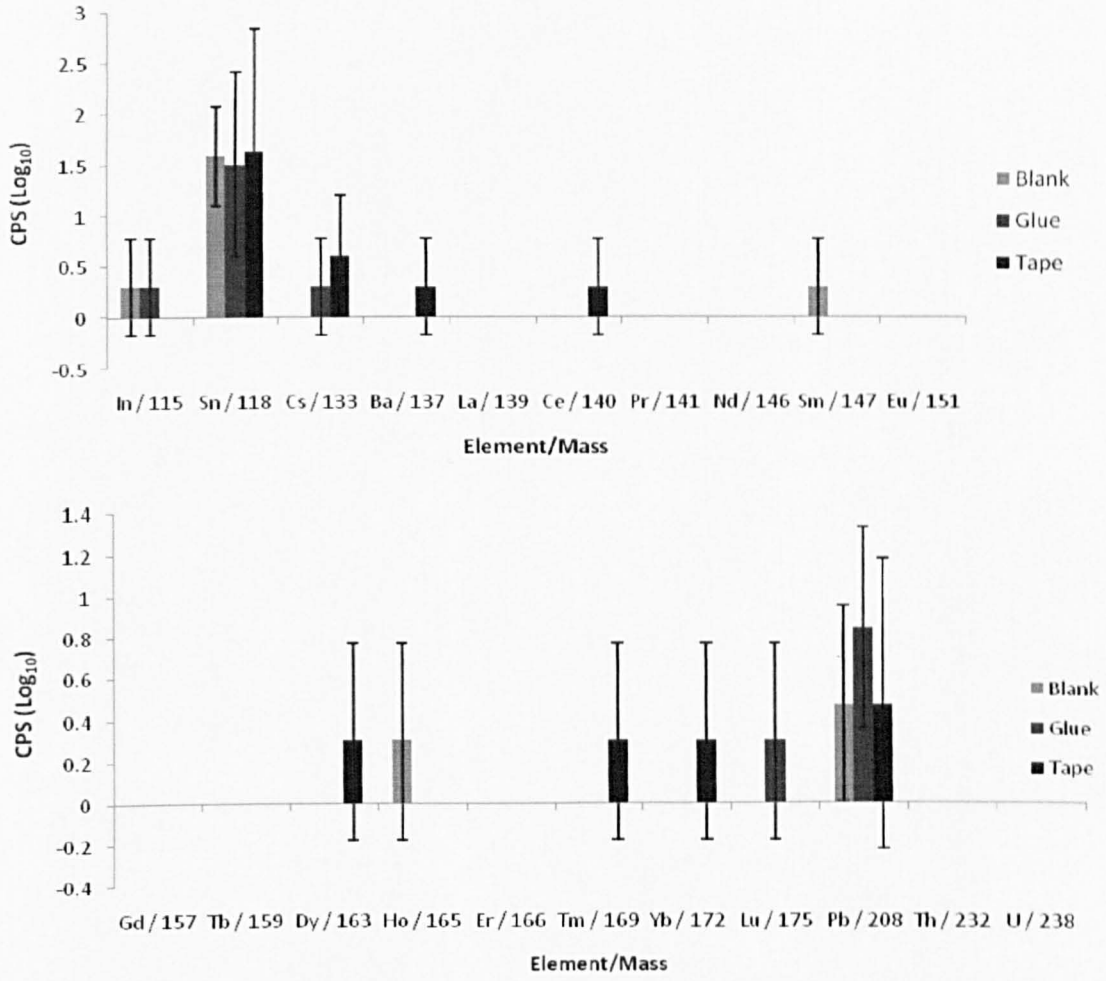


Figure 1. Average counts per second (CPS)($\log_{10}$)(±1SD) of each isotope in the gas blanks (n=3), glue (Araldite) (n=3) and tape (No More Nails Tape) (n=3), as determined by LA-ICP-MS (n=number of measurements made). Data for the glue and tape were not blank corrected.