A holistic stock analysis of Albacore tuna (*Thunnus alalunga*) from the North Atlantic Ocean and Mediterranean Sea

A holistic stock analysis of Albacore tuna (*Thunnus alalunga*) from the North Atlantic Ocean and Mediterranean Sea

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DECLARATION OF PhD THESIS

I hereby declare that the work in this thesis is my own and that it has not been used to obtain a degree within this Institute of Technology or elsewhere.

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Supervisor Name	**Date**

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Diolch i chi fy nheulu, yn arbenig mam a dad ac Brynmor fy mrawd, am eich cefnogi trwy y sefyllfa hyn. Ac eich caredigrwydd ata'i trwyddo yr addysg i gid, ac yn ystod y tymor diwethaf.

I'r ci bach bendegedig, cydymaith cyson yn yr amseru dda ac yr amseru wael. Ma bywyd yn rhy fyr. Gorffwys yn heddwch, Siâny.

Diolch yn fawr!

Carys

"We never think entirely alone: we think in company, in vast collaboration; we work with workers of the past and of the present. [In] the whole intellectual world … each one finds in those about him [or her] the initiation, help, verification, information, encouragement, that he [or she] needs".

A. G. Sertillanges

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Summary

Albacore tuna (*Thunnus alalunga*, Bonnaterre, 1788) is a highly migratory scombrid with a cosmopolitan distribution inhabiting all the worlds' ocean, in both tropical and temperate regions. Albacore juveniles and adults are highly exploited, with the main commercial fisheries in the North East Atlantic Ocean and the Mediterranean Sea targeting juvenile feeding aggregations. The life history of this species is poorly known and very little is understood regarding the stock origin of the exploited fish. Current management assumes a single panmictic stock in the North Atlantic with the Mediterranean as a separate management unit; previous studies have identified potential heterogeneity within the North Atlantic stock. Uncertainties are evident in age estimations and the interpretation of growth increments of this species; this presents problems for stock assessment which is based on an age-structured model. This study evaluates age estimation and stock discrimination techniques (otolith chemistry, otolith shape and genetics). It addresses the potential evidence of stock heterogeneity. This study provides new insight into stock structure in the North Atlantic Ocean and Mediterranean Sea.

A comparative exercise was undertaken to assess bias and precision in inter- and intra-reader age estimations. Dorsal spines are the preferred aging structure in albacore tuna. The level of precision obtained using spines and alternative structures (scales and otoliths) for age estimation was assessed. Inconsistencies in interpretation between structures and between age-readers may introduce error into age data used for stock assessment. Linear bias was detected among readers for all structures, with readers systematically over- or underestimating age relative to each other. Agreement of age estimates derived using different structures from the same fish was low, and

scales appeared to provide lower estimates of age than spines. The vascularisation of the core region of the spine can affect accurate age assessments in older fish. The readability of the first annual increment in the dorsal spine was tested. The first annulus was consistently identified in albacore aged two to five. Inter-cohort differences in growth were identified. The study highlighted the need for further inter-calibration of albacore tuna ageing methodologies. Additional study is required on age-specific growth rates within different albacore cohorts.

Otolith chemistry can be used as a stock descriptor in fish species. To ensure that results from albacore otolith and dorsal spine chemistry are robust to contamination, an investigation of post-mortem contamination and effective removal was undertaken using laser ablation inductively coupled plasma mass spectrometry (LA ICPMS). Magnesium and strontium were shown to be relatively robust to post-mortem effects at concentrations of 50ppm and 200ppm, respectively. Cleaning contaminated structures in ultra pure water and hydrogen peroxide was not effective at removing contamination from either structure. Washing the otoliths and spines in nitric acid successfully removed post mortem contaminants. Dorsal spines and otoliths were shown to be poorly correlated; therefore this study does not support the use of spines as an alternative to otoliths in trace elemental analyses of albacore tuna.

Cross-species amplification success of microsatellite loci developed for bluefin tuna were tested in albacore tuna. Amplification success was 100%. Species specific information was produced for 33 microsatellite loci. Stock heterogeneity was investigated in albacore tuna using twelve polymorphic microsatellite loci. Pairwise F_{ST} values and exact tests showed that the South Pacific, North Atlantic and Mediterranean stocks were distinct from one another. Heterogeneity was observed for the first time between samples from the western and central Mediterranean Sea.

Evidence of temporal stability in samples collected in 2006 and 2007 from fisheries operating in the central Mediterranean Sea was provided. Weak seasonal genetic structuring was observed in the North East Atlantic. Differences were attributed to samples collected in either the early or late fishing season. Observed genetic structure was proposed to be related to differences in migrations patterns with separate sub-populations moving to feeding grounds in either summer or autumn.

A variety of stock discrimination techniques applied differentiated between samples taken from two separate stocks, the North Atlantic and Mediterranean. North Atlantic fish were larger in length at age and weight at a given length than Mediterranean fish. The highest classification success was observed using microsatellite DNA data. Otolith microchemistry data showed moderate differences between stocks although otolith chemistry is unlikely to be a useful method for characterising spawning areas in albacore. Poor stock separation was observed using otolith shape variables. At this time it appears that microsatellite DNA data provides the most powerful discriminatory power for separating individual albacores between the North Atlantic and Mediterranean stocks.

Chapter 1: Introduction

1.1 General Information on Albacore Tuna

Albacore (*Thunnus alalunga*, Bonnaterre 1788) like other tuna species is highly migratory. It inhabits tropical and temperate waters of all oceans and is considered one of the most important commercial tuna species in world landings, being valued for its white meat (Shomura *et al.*, 1993). The species moves in schools of similar sized fish, which can include other tunas such as skipjack, yellowfin and bluefin. Maximum fork length (L_F) is presumed to be 127cm with the largest angling record of a single fish of 123cm L_F and weight of 40kg caught in the Canary Islands in 1977 (Collette and Nauen, 1983). Length at maturity differs slightly in each ocean but full maturity is believed to be reached at 90 to 97 cm or 5 years old (Santiago and Arrizabalaga, 2005). Fecundity is estimated to be between 0.8 and 2.6 million eggs per spawning (Shomura *et al.*, 1993). Spawning takes place in the summer months; northern hemisphere fish have an assumed birth date of 1st May and southern hemisphere fish are assigned a birth date of 1st November (Beardsley, 1969; Piccinetti and Piccinetti Manfrin, 1993; Shomura *et al.*, 1993; Chen *et al.*, 2005; ICCAT, 2007). The selections of birthdates in fish are in effect estimations of breeding times in adult fish but can vary with seasonality (Wright and Gibb, 2005). Assigned birthdates of fish are required from a management perspective for estimating spawning stock biomass at the height of the breeding season and for attributing a definitive yearly age to an individual fish which as used in age based models (Fournier *et al.*, 1998)

1.2 Tuna Migration Patterns

Transoceanic migration patterns of albacore have been determined from catch per unit effort (CPUE) statistics from fishery data over time in 5° squares across a range of longitudes and latitudes (Beardsley, 1969; Shomura *et al.*, 1993; Chen *et al.*, 2005), tag returns (Arrizabalaga *et al.*, 2003) and analysis of parasitic faunal loads (Jones, 1991). Migrations occur between summer and winter feeding areas in juveniles, and summer spawning and winter feeding areas in adults. Juveniles occur more frequently in temperate waters compared to the subtropical ranges of adults. Spawning occurs in the summer months of both hemispheres. Figure 1.1 shows the major migration pathways of albacore tunas.

Migration patterns of albacore can be subdivided into sub-adult and adult migrations. Sub-adults move from summer (temperate) to winter (sub-tropical to tropical) feeding grounds in the warmer surface waters, whilst adults once mature move into deeper waters (250-300m) and continue migration between feeding and spawning grounds in the tropical and sub-tropical waters (Beardsley, 1969; Yoneta and Saito, 1973). Atlantic albacore show transoceanic movements from east to west and back again in both hemispheres (Beardsley, 1969). The highest concentrations are found in the Sargasso Sea and off northern Venezuela in late spring and early summer in the northern hemisphere, and off the eastern coast of Brazil in the southern Atlantic in the austral spring and summer where spawning is believed to take place (Beardsley, 1969). A smaller assemblage of larger fish congregate around western South Africa in the austral winter but leave this area, possibly moving into the Indian Ocean in summer. Small albacore (>8kg) in the northern Atlantic appear off Cape Finisterre, Spain in June and these fish reside around the Bay of Biscay until returning to Cape Finisterre between October and November. Between December and May these animals possibly move southwest to mix with larger albacore around 35°N and 35°W (Beardsley, 1969). It is accepted that there are no trans-equatorial movements of albacore (ICCAT, 1996).

Distribution of albacore in the Mediterranean is discontinuous with the highest concentrations in the Tyrrhenian, Ionian, Adriatic and Aegean Sea (Megalofonou, 2000). Migrations take place in a north to south manner from feeding/fishing grounds in the north to the spawning areas in the south. Piccinetti and Piccinetti Manfrin (1993) showed that the highest concentrations of albacore larvae were found off the coast of North Africa. Tagging surveys have shown that migration from the North Atlantic to the Mediterranean Sea and vice versa does occur, but the number of confirmed tagged displacements is statistically a very low percentage of the total number of tagged fish (Arrizabalaga *et al.*, 2002; Arrizabalaga *et al.*, 2003).

1.3 Tuna Fisheries

Five methods are used to catch albacore tunas; long-lining, live-bait fishing, trolling, purse-seining and pelagic pair trawling. FAO[1] statistics show albacore catches in 15

[1] Food and Agriculture Organization of the United Nations

FAO Fishing areas, with the peak of catches of 1.2 million tonnes in 1993 (URL 1). The largest catches are taken by Far East countries (URL 1).

In the Atlantic, the surface fishery is exploited by vessels from Spain, France, UK and Ireland by bait-boats, trolling and pair trawling (ICCAT, 1996). Albacore are also caught on the high seas by vessels from Portugal (bait boats) and USA (recreational angling). There is also a small surface fishery persecuted by South African boats in the South Atlantic off the coast of South Africa and Namibia. Long-line fisheries targeting adult fish along lower latitudes are dominated by Far Eastern fleets. Commercial fishing operations in the North East Atlantic target aggregations of juvenile and sub-adult fish on summer feeding grounds in the Bay of Biscay (ICCAT, 1996).

The Irish fishery developed in 1990 using driftnets and reached a peak of 4,858 tonnes in 1999 with over 30 vessels being employed. Since 1999 annual landings have steadily decreased to a 10 year low of 175 tonnes in 2004. This is primarily due to the 2001 ban on driftnet fishing (Council Regulation (EC) No 1239/98[2]). Alternative fishing techniques of pelagic pair trawling, long-lines and mechanised trolls were investigated by BIM[3] in order to address the negative repercussions of the driftnet ban (Anon, 2000). Pelagic trawls emerged as the most promising alternative with 20 vessels using this method annually. However whilst Total Allowable Catch (TAC) has increased over the last few years (set at 3,216 tonnes until the end of 2005; increased in 2006 to 5,679 tonnes and again in 2007 to 8,236t) the actual landings have decreased (Anon, 2007) (Figure 1.2).

1.4 Tuna Stock Management

Each stock (identified as the total exploitable population within a single ocean basin) is regulated by different commissioning bodies in a framework of Regional Fishery Management Organizations (RFMO). There are five RFMOs for tunas: the International Commission for the Conservation of Atlantic Tunas (ICCAT) (URL 2), which covers the North and South Atlantic with 43 contracting parties; The Inter American Tropical Tuna Commission (IATTC; (URL 3) 16 member states; the Indian

[2] Council Regulation (EC) No 1239/98 of 8 June 1998 amending Regulation (EC) No. 894/97 laying down certain technical measures for the conservation of fishery resources.
[3] Bord Iascaigh Mhara

Ocean Tuna Commission (IOTC (URL 4) 25 member states; Commission for the Conservation of Southern Bluefin Tunas (CCSBT www.ccsbt.org (URL 5) 8 member states), whose primary concern is the Southern Bluefin tuna; Western and Central Pacific Fisheries Commission (WCPFC (URL 6) 25 member states. All five RFMOs are responsible for the management and conservation of tunas and tuna-like species (and to some extent by-catches) taken by tuna-fishing vessels within the various oceans and adjacent seas.

Albacore tuna in the Atlantic are treated as two separate stocks, North Atlantic and South Atlantic, the separation being at 5°N for assessment purposes (ICCAT, 1996; 2007). The Mediterranean population is also regarded as a separate stock and forms a distinct management unit (ICCAT, 1996). The separation of North Atlantic South Atlantic and Mediterranean stocks is based on differences in distribution, the occurrence of distinct spawning grounds in the three areas (Beardsley, 1969; Piccinetti and Piccinetti Manfrin, 1993) and the results of tagging experiments, which have shown minimal movement between the areas and differences in growth and morphological characteristics (Arena, 1990; ICCAT, 1996; Megalofonou, 2000).

The current state of the albacore stock in the North Atlantic is evaluated by ICCAT. Recent stock assessments have indicated that the spawning stock size has declined and is currently a quarter of peak levels experienced in the 1940's (Anon, 2009). The maximum sustainable yield (MSY) is currently estimated at 30,200t (Anon, 2009) but the total allowable catch (TAC) in this fishery in recent years (up to 2007) has been higher at 34,000t. Current management proposals include reducing the TAC to 30,200t i.e. the current estimated MSY (Anon, 2009), and limiting the number of fishing vessels (Anon, 2008). In 2006 yield exceeded TAC and MSY at 36, 077t (Anon, 2008). Yield has been shown to decrease in the last stock assessment based on catches in 2007, although this is primarily due to a decrease in landings by the long-lining fleet (Anon, 2009). ICCAT project that if catch levels persist above 30,000t then the stock will not recover from these overfished conditions (Anon, 2009). The current state of the Mediterranean stock is unknown as no stock assessment has ever been undertaken in this region (Anon, 2008; 2009).

1.5 Methods of Tuna Stock Analysis

Many marine fish species are characterised by large population sizes, strong migratory behaviour, high fecundity, and pelagic eggs and larvae that are subject to passive transport by ocean currents, all factors which tend to reduce the rate of development of genetic partitioning among localised populations (Cadrin *et al.*, 2005). Nevertheless, in many cases, discrete subpopulation structure is still observed in many marine species. Stock complexity can arise through heterogeneity in the timings, duration and location of spawning (Brophy and Danilowicz, 2002; Cadrin *et al.*, 2005), salinity and temperature discontinuities between water bodies (Jørgensen *et al.* 2005), oceanographic gyres favouring larval retention or separation along an oceanographic front (Was *et al.*, 2008) and natal homing to specific locations (Thorrold *et al.*, 2001). Differences caused by oceanographic separation of population components can result in easily identifiable separate stock management units (e.g. herring in the Irish and Celtic Seas, Iles and Sinclair, 1982). However in albacore tuna, although separate stock components have been proposed in the North Atlantic (Hue, 1979; 1980; Arrizabalaga *et al.*, 2004) and the distribution of Mediterranean albacore is know to be discontinuous (Piccinetti and Piccinetti Manfrin, 1993; Piccinetti *et al.*, 1997; Megalofonou, 2000), both are treated as whole basin stocks for management (ICCAT, 2009). Stock complexity has been shown in the sympatric species of bluefin tuna (*Thunnus alalunga*) in the same geographic areas (Carlsson *et al.*, 2006; Rooker *et al.*, 2007). The potential for complexity in albacore tuna is interesting due to the highly migratory nature of this species, which shows potential differences in migration patterns and spawning timings within the known stocks (Hue, 1979; 1980; Piccinetti and Piccinetti Manfrin, 1993; Piccinetti *et al.*, 1997; Oritz de Zárate and Cort, 1998; Megalofonou, 2000; Arrizabalaga *et al.*, 2004).

The stock of albacore in the North Atlantic is treated as a single unit (ICCAT, 1996) but has a high degree of dispersal potential and shows distinct intra-oceanic migration patterns between feeding and spawning areas (Beardsley, 1969). Some evidence of structuring within the North Atlantic albacore is provided by observations of separate trophic migration routes proposed in Oritz de Zárate and Cort (1998) and protein electrophoresis studies (Hue, 1979; 1980). The current life history parameters of albacore tuna are poorly known (Santiago and Arrizabalaga, 2005). The current

stock assessments and management advice are based on an age structured model (ICCAT, 2007). As the life history of albacore is acknowledged as being poorly known it is necessary to improve the understanding of the biology and stock structure of albacore. Misinterpretation of life history parameters and inefficient management can have serious repercussions on a fishery. For example, orange roughy (*Hoplostethus atlanticus*) were initially assumed to be quick growing with early maturation and high fecundity, it is now understood that the life history parameters are in fact opposite, i.e. slow growing with late onset of maturity, however this information was too late to counteract the high exploitation levels of this fish in 1970's, which has led to the crash of the fisheries around Australia and New Zealand (Clark *et al.*, 2000). As albacore is managed as a single stock it is essential to prove or disprove this assumption, fishery managers must ensure that the stock, or stocks, are managed effectively to retain a healthy sustainable fishery.

Basic biological data is essential to understand the dynamics of any population; this includes parameters such as size measurements (e.g. length, height, weight), age estimates and growth rates (Haddon, 2001). This data is especially crucial for estimating the productivity of fish stocks and monitoring the impacts of exploitation (Begg *et al.*, 1999b). Observed variation in the biological data between any two populations occupying different geographic areas or niches can be attributed to a variety of different factors working in combination such as environmental influences (temperature and salinity), food availability and genetics. Life history parameters can provide a phenotypic marker of stock differences and can be used in combination with other methods such as genetic stock identification to elucidate stock structure (Begg *et al.*, 1999b).

Stock composition (i.e. the number of distinct populations) can be inferred from morphometric characteristics of otoliths (ear stones) (Brophy and Danilowicz, 2002; Cardinale *et al.*, 2004; Stransky, 2005) and other meristic traits (Schaefer, 1991). Otolith shape is species specific (L'Abee-Lund, 1988) and has been shown to vary between populations within a species (Bird *et al.*, 1986; Castonguay *et al.*, 1991; Campana and Casselman, 1993; DeVries *et al.*, 2002). Differences in elemental uptake can produce otolith chemical signatures or "elemental fingerprints" (Thresher, 1999), which if distinct between geographic areas, can be used to identify origin of fish (Brophy *et al.*, 2003; Shuford *et al.*, 2004) or trace population movements (Campana *et al.*, 1994; Begg *et al.*, 1998; Rooker *et al.*, 2003). A range of parameters

used in conjunction can reveal a more accurate illustration of the stock composition and fluctuations (Waldman, 1999; Smith *et al.*, 2002; Miller *et al.*, 2005). Otolith micro-chemistry has successfully been utilized in other tuna species to elucidate stock structure (Proctor *et al.*, 1995; Rooker *et al.*, 2001a) and has been used to differentiate between the origins of mixed tuna stocks (Rooker *et al.*, 2003). However neither otolith chemistry nor otolith shape analysis has been applied to albacore tuna.

Since their inception in the 1960s molecular markers have been widely used to detect genetic structure in marine fish species (Cadrin *et al.*, 2005) and can provide information for management decisions when genetically differentiated populations are identified within the targeted species. Approaches applied to the elucidation of stock structure in albacore include the analysis of blood proteins, allozymes, mitochondrial DNA (mtDNA) and nuclear DNA. Studies to date have concentrated on assessing genetic differences between albacore populations within the major ocean basins. Significant differences between Indo-Pacific and Atlantic populations have been reported by Chow and Ushiama (1995) using PCR-RLFP of the mtDNA ATPase gene. Results from blood group and lectin analysis indicate that populations of albacore from different areas within the Mediterranean Sea are distinct from those in the North Atlantic Ocean (López-Rodas *et al.*, 2002; Arrizabalaga *et al.*, 2004). Microsatellites as highly variable non-coding regions of nuclear DNA are a powerful tool for detecting genetic variation between populations (Ferguson, 1994; O'Connell and Wright, 1997). Takagi *et al.* (2001) used micro-satellites to show that populations of Atlantic albacore tuna are distinct from populations from the Indian and Pacific Oceans, as well as being distinct between the North and South Atlantic basins.

1.6. Outline of Thesis and Summary of Hypotheses

Stock analysis of albacore tuna is based upon a catch at age model, assuming a single stock occurs in each ocean basin (ICCAT, 2009). The main purpose of this study was to investigate the single stock hypothesis using a holistic approach; combining information from a number of different methods. These include traditional age and growth metrics, morphometric parameters and more modern methods such as microchemistry of otoliths and microsatellite DNA analysis. The study also

investigates the robustness of methodologies used in stock assessment and stock identification. Albacore stock structure was investigated within the North Atlantic Ocean and Mediterranean Sea. Fish were sampled from the Irish fishery (which is concentrated in the North Atlantic Ocean to the South West of Ireland and into the Celtic Seas and Bay of Biscay) and from the Mediterranean Sea: the population that is geographically closest to the North Atlantic stock. ICCAT currently regards albacore in the North Atlantic as a single stock (ICCAT, 2007) despite recent evidence indicating intra-regional structure (López-Rodas *et al.*, 2002; Arrizabalaga *et al.*, 2004).

The following thesis is presented as five main chapters followed by a discussion of findings with concluding remarks. Each chapter is intended as a stand alone piece of work, these have been written in the format of articles intended for peer reviewed publication.

Chapter 2 investigates the precision of age estimations from an inter-laboratory exchange. The precision of age estimates undertaken by six scientists from four international laboratories are compared for the three main calcareous structures (otoliths, scales and dorsal fin ray spines) used in age assessments for albacore tuna. Ageing precision is assessed between age readers, between structures and within readers as repeated age estimations of the same structure (Beamish and Fournier, 1981; Campana *et al.*, 1995; Campana, 2001). The presence or absence of dorsal spine vascularisation and its effect on ageing is investigated. Back-calculated lengths at age are compared to assess the methods of estimating length at earlier stages in the fish's life history. The hypotheses tested in this chapter are:-

- That there is no difference in the ages assigned to an individual albacore by one reader against another, i.e. there is total precision between age estimates
- That the calcareous structure used to age a fish makes no difference to the assigned age of that fish
- That there are no differences in growth rates or weight-at-length of albacores from the North Atlantic Ocean and Mediterranean Sea

Prior to investigating the use of trace elements of otoliths and dorsal fin spines as stock descriptors by assessing their use in elucidating stock structure and migration patterns in albacore tuna, an assessment of the likelihood of contamination and ease of removing post-mortem contamination of the structures is undertaken and described in

chapter 3. The effectiveness of three decontamination methods are assessed in calcareous structures analysed by laser-ablation inductively coupled plasma mass spectrometry. The correlation between otolith and spine micro chemistry is investigated. The null hypotheses tested here are:-

- That otoliths and dorsal spine microchemistries are not subject to post-mortem contamination when used in analysis by laser ablation inductively coupled plasma mass spectrometry (LA ICPMS)
- That the method of decontamination makes little or no difference to the level of contaminants found on the otolith or dorsal spine surface when analysed by LA ICPMS
- That there is no difference in the microchemistry between the otolith and dorsal spine from a single fish.

Microsatellite DNA markers are the most commonly used marker used to elucidate population structure in fisheries. No microsatellite loci have been developed specifically for albacore tuna. Therefore the characterizations of 33 bluefin tuna microsatellite loci that are cross-amplified in albacore are presented as a short communication in Chapter 4. A single study using microsatellites in albacore has been published (Takagi *et al.*, 2001), indicating differences in albacore between the oceans. Other less variable genetic markers have been used to show differences between the North Atlantic and Mediterranean albacore (Arrizabalaga *et al.*, 2004; Viñas *et al.*, 2004; Nakadate *et al.*, 2005). Therefore the null hypothesis tested in this chapter was:-

- That there are no differences in the properties of microsatellites developed for bluefin tunas, when applied to albacore tuna, i.e. cross-amplification

The study in chapter 5 uses microsatellites to investigate differences in samples within the North East Atlantic Ocean and Mediterranean Sea across three sampling years, where the hypothesis tested was:-

- That there are no differences in the microsatellite DNA between or within samples of albacore from the North Atlantic Ocean or Mediterranean Sea

Chapter 6 is a multi-disciplinary study which combines a number of different phenotypic stock discrimination methods to distinguish between samples of albacore, which show genetic variation. The stock discrimination approaches used are: a comparison of growth parameters, analysis of microchemistry variation within the

natal regions and edge of otoliths, and an assessment of otolith shape variation. The genetic distance between samples and classification success using microsatellite DNA markers are also assessed. The resolving power of the different methods is compared. The main hypotheses tested in the chapter are:-

- That there is no difference in the microchemistry of otoliths or dorsal spines between fish of North Atlantic or Mediterranean origin.
- That there are no differences in otolith shape between albacore of North Atlantic and Mediterranean origin
- That there are no differences in the ability of one method to distinguish stock origin over another method.

A final synthesis and conclusions based on the main findings of the thesis are presented in Chapter 7; ideas for potential future research are also discussed.

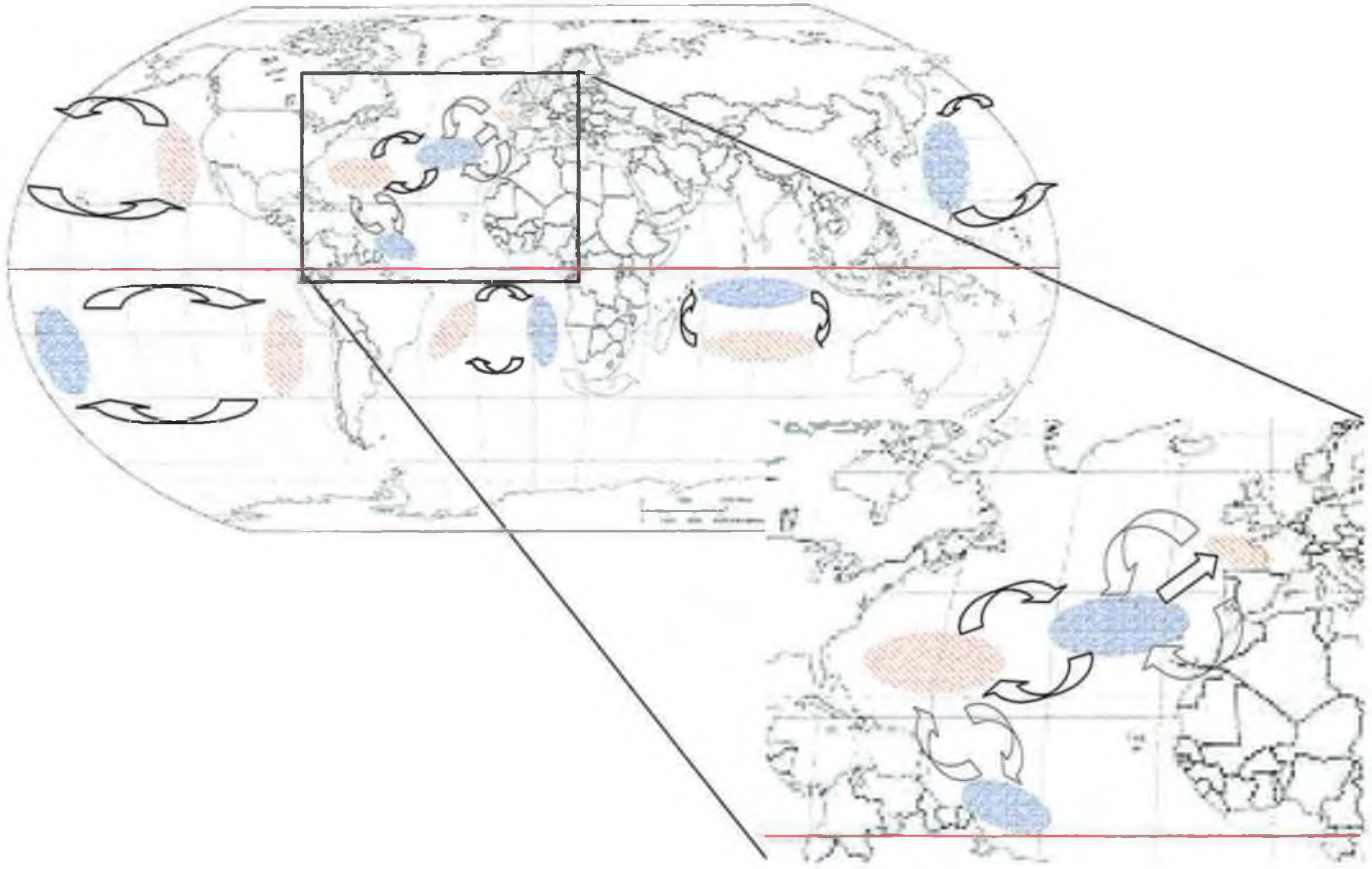

Figure 1.1. Diagrammatic representation of the proposed migration routes of albacore in the major ocean basins (red diagonal lines = summer feeding grounds, Blue = winter feeding grounds) (Beardsley, 1969; Jones, 1991; Shomura *et al.*, 1993; ICCAT, 1996; Chen *et al.*, 2005).

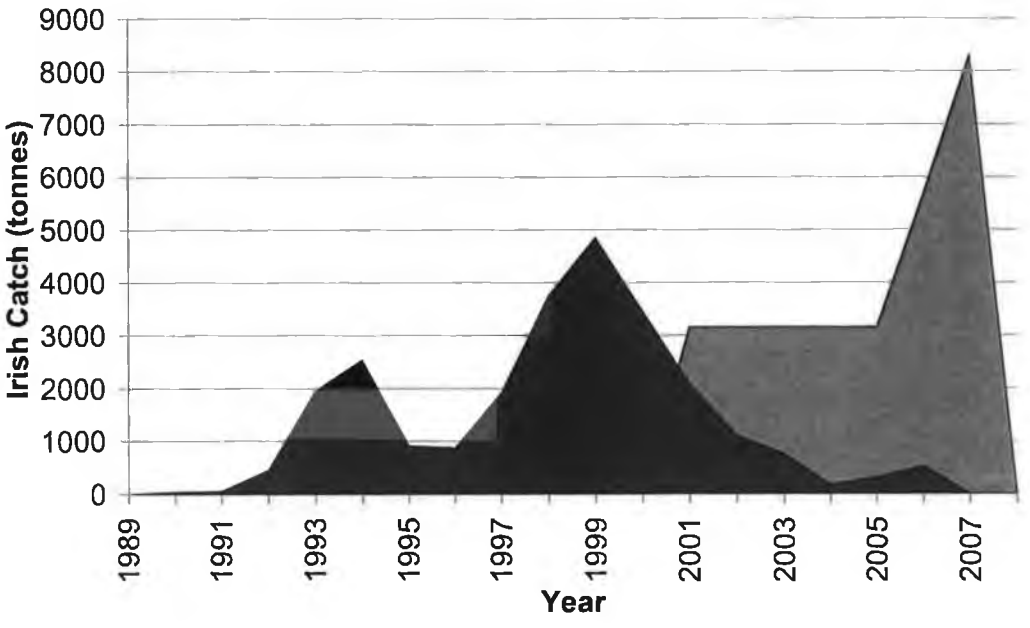

Figure 1.2. Irish catch data (black) versus annual Irish quota (grey) (data from (URL 2) and (Anon, 2007)).

Chapter 2: An Inter-Laboratory Assessment of Precision in Age Estimation of Albacore Tuna *Thunnus alalunga* (Bonnaterre, 1788)

2.1. Abstract

Age estimates for albacore tuna are derived using enumeration of growth increments in spines, scales and otoliths. Inconsistencies in interpretation between structures and between laboratories may introduce error into the age data available for stock assessment. A comparative age estimation exercise was carried out to assess bias and precision of age estimates between readers and between structures. Six scientists from four laboratories, with varying levels of experience in albacore age estimation participated in the study. Images of the otoliths, scales and spines from 84 albacore tuna collected from the North East Atlantic Ocean and the western Mediterranean Sea were distributed to each reader. When the age estimates of an experienced reader were compared with those of an inexperienced reader who they had trained, moderate levels of precision were observed (mean % coefficient of variation 11.2, 7.7 and 6.5 for otoliths, scales and spines, respectively). However, the wider comparison across laboratories yielded poor precision (% coefficient of variation 35.7, 25.7 and 24.0 for otoliths, scales and spines, respectively). Linear bias was detected between readers for all structures, with readers systematically over- or underestimating age relative to each other. Analysis of variance indicates that inter-reader precision is higher for Mediterranean albacore than for Atlantic albacore. Agreement of age estimates derived using different structures from the same fish was low, and scales appeared to provide lower estimates of age than spines. Coefficient of variance of repeated age estimations ranged from 0 to 43.2% and percentage agreement ranged between 7 and 100%. These results highlight the need for further inter-calibration of albacore tuna ageing methodologies.

Re-absorption or vascularisation of the core region of the spine may obscure the first increment in older fish, leading to underestimation of age. Comparison of the size of the first annulus and the width of subsequent growth increments between age groups suggests that in this study, age estimates were not affected by misinterpretation of the first annulus in older fish. However, there was evidence of enhanced survival of faster growing fish. Length at age 1 was estimated using five back-calculation methods and compared to theoretical estimates from the Von Bertalanffy equation. Visual comparison indicated that the back calculated length at age 1 was consistent with predicted length at age 1. It is proposed that back-

calculation models that incorporate an age-effect are most appropriate for albacore tuna as the relationship between spine diameter and fish length was observed to vary between age groups and between cohorts.

Keywords: Albacore, *Thunnus alalunga*, Ageing, Growth, Precision, Bias, Scales, Spines, Otoliths.

2.2. Introduction

Accurate assessment of an exploitable fish stock depends on the accurate and precise estimation of catch-at-age (Campana, 2001). Age is estimated by counting periodic growth increments in calcified structures at an annual or daily scale (sclerochronology). For most fish in temperate waters these growth marks appear as opaque and translucent deposits, which represent periods of fast and slow growth, respectively. The structures that have proved useful in the ageing of teleost fish are scales (FitzGerald *et al.*, 1997; Abecasis *et al.*, 2008), vertebrae (Prince *et al.*, 1985; Alves *et al.*, 2002), fin rays (Cass and Beamish, 1983), cleithra (Casselman, 1983), opercula (Baker and Timmons, 1991) and otoliths, although it is the latter that is applied over the broadest age range in many species (Secor *et al.*, 1995). Campana and Thorrold (2001) reviewed the available literature and found that most fish age estimations in 1999 were made using scales and otoliths.

The process of age estimation is affected by two main sources of error; firstly, error associated with the type of structure under examination. It is possible that the entire axis within a given structure does not show a complete growth record (Campana, 2001), or that the growth marks are formed at irregular intervals. This produces inaccuracies in the age estimates obtained. The second source of error is due to imprecision in the age estimates obtained from repeated readings of the same structure. This interpretation error can be biased or random, and can result in variation in age estimates between readers and between laboratories (Campana and Moksness, 1991). Therefore, when evaluating methods of age determination, two terms are of interest; firstly "precision" i.e. the percentage agreement or variability between readings of the same specimen by the same or different age-readers. Secondly, "accuracy" describes the comparison of ages generated by age-readers with the "true" age for specimens of a known age (Kimura and Lyons, 1991). Only through tag-release recapture studies or known age fish (i.e. reared) can the true age and thus accuracy of age estimations of a population be validated (Beamish and McFarlane, 1983). In the absence of known-age reference collections ageing consistency is the best that can be achieved (Campana *et al.*, 1995). Bias in precision and accuracy of age estimates can be both relative: the systematic over or under estimation of age compared to the modal age, or absolute: the systematic over or

under estimation of age compared to true age (Eltink *et al.*, 2000). This study addresses relative bias in precision of age estimates.

The species under investigation here is albacore tuna *Thunnus alalunga*, (Bonnaterre, 1788). Albacore is a highly migratory species of tuna, with a pan-oceanic distribution, inhabiting both tropical and temperate waters. It is considered one of the most important commercial tuna species in world landings, being valued for its white meat (Shomura *et al.*, 1993). The life span of albacore is assumed to reach 15 years (Oritz de Zárate *et al.*, 2005). The calcified structures used to estimate age in albacore include scales (Megalofonou *et al.*, 2003) spines (González-Garcés and Farina-Pérez, 1983; Santiago and Arrizabalaga, 2005) otoliths (Fernández, 1992) and vertebrae (Fernández, 1992). Dorsal spines have emerged as a preferred structure (ICCAT, 2003) due to relative ease of availability and preparation, however ageing protocols using this structure must take into account issues such as multiple banding (Compean-Jiménez and Bard, 1983; Santiago and Arrizabalaga, 2005; Oritz de Zárate *et al.*, 2007a) within each annulus, and the vascularisation/reabsorption of the core region. Ageing using otoliths has been proposed as an alternative to ageing using dorsal spines (ICCAT, 2003) and daily increment analysis in otoliths has been shown to be effective in albacore (Laurs *et al.*, 1985; Lee and Yeh, 2007) and other species of tuna (Wild *et al.*, 1995; Itoh and Tsuji, 1996; Tanabe *et al.*, 2003; Doray *et al.*, 2004; Stéquert and Conand, 2004) as a method of validating true age. However any data obtained from these age validation studies must be transferable to other structures which may be used as the preferred ageing structure.

The catch-at-age matrix currently used in the assessment of Atlantic albacore is derived using likelihood based analysis (MULTIFAN) of the catch-at-length composition of the fishery (Santiago and Arrizabalaga, 2005). Although the statistical approach has the advantage of objectivity, variability in mean length-at-age is not accounted for and can affect the precision of parameter estimation (Fournier *et al.*, 1998). In contrast, ages estimated from periodic bands on calcified structures (otoliths, scales, spines and vertebrae) reflect individual variation in growth rates and are valuable for resolving catch-at-age estimation and validating MULTIFAN analysis (Oritz de Zárate *et al.*, 2007b). Age estimates from all the structures are affected by a degree of uncertainty. Reports of the timing of increment formation are sometimes contradictory, particularly in relation to the first increment (Santiago and Arrizabalaga, 2005). Validation studies using mark-recapture techniques (Oritz de

Zárate *et al.*, 1996) and daily increment counts in otoliths (Lee and Yeh, 2007) are necessary to clarify the relationship between growth increments and absolute age, and to ensure the accuracy of age estimates. Precision in age estimation is of paramount importance and poor reproducibility within and between readers, laboratories and ageing techniques will increase the margin of error associated with the parameters estimated using the catch-at-age matrix. Systematic ageing error can lead to misinterpretation of spatial and temporal growth patterns and can influence stock assessment outputs (Tyler *et al.*, 1989; Reeves, 2003). The potential influence of inconsistencies in ageing procedures on catch-at-age data is highlighted in the International Commission of the Conservation of Atlantic Tunas (ICCAT) albacore stock assessment report (ICCAT, 2003). Regional comparison of ageing criteria and exchange of samples is recommended, and the investigation of alternative structures such as otoliths for ageing is suggested (ICCAT, 2003).

The objective of this comparative age estimation study is to compare ageing criteria across laboratories and to assess bias and precision of age estimates obtained using three calcified structures (otoliths scales and spines). Six scientists from four laboratories, with varying levels of experience in albacore age estimation participated in the study. Age estimates are compared between readers and between methods, statistically and graphically, and the reproducibility of age estimates is assessed. Vascularisation or re-absorption of the core of spines is a problem encountered in age estimations. The size of the first annulus and the width of subsequent growth increments are compared between age groups to ascertain if the first annulus was consistently identified in 2 to 5 year old fish. Age estimates from spines are used to back calculate mean length-at-estimated ages. Estimates obtained from five different back calculation methods are compared with estimated length-at-ages from the Von Bertalanffy growth parameters used in albacore stock assessments (ICCAT, 2003).

2.3 Materials and Methods

Sample Collection and Preparation

Albacore were obtained from Irish commercial vessels fishing along the Porcupine Bank in the North Atlantic (Bay of Biscay) between 2005 and 2007 (n=650); and Mediterranean fish were sourced from fish merchants in the Balearic Sea during the summer of 2005 and from the Tyrrhenian Sea in 2006 and 2007 (n=152) (Figure 2.1). Length and weight measurements were taken for each fish. The first dorsal spine ray and scales from under the pectoral fin were removed from each fish and stored frozen soon after collection. Sagittal otoliths were removed from the head, cleaned and stored dry in plastic tubes at room temperature.

The scales were re-hydrated by dipping in water and cleaned of mucus by rubbing between the fingers. Suitable scales were placed on a glass slide with a drop of water and viewed under a binocular microscope (Megalofonou *et al.*, 2003). Eight scales per fish were selected on the basis of clarity and photographed at 2.5x magnification.

The first spine from the first dorsal fin ray was embedded in Buehler Epo-thin® low viscosity epoxy resin. Three sections of 1mm in thickness were made using a Buehler Isomet® Low Speed Saw with Buehler Iso-Cut Fluid as a blade lubricant, following protocols later presented by Oritz de Zárate *et al.* (2007a). The sections were placed on a slide and digital images of each section were taken at 1.6x magnification.

One sagittal otolith was chosen at random and embedded in a 1.5cm^2 block of resin, as above. Four 0.75mm transverse sections were made on the Buehler Isomet® Low Speed Saw (Doray *et al.*, 2004). Each section was polished on 600, 2500 and 4000 grit silicon carbide wet-dry polishing paper with 3µm diamond paste; the final polish was on a Buehler micro cloth. Sections were mounted on a glass slide with PolyMount® and covered with a glass slip. Digital images of each section were taken at 4x magnification.

All digital images were taken using a stereoscopic microscope with a digital camera and PC interface. For each of the three structures, the same magnification and light intensity parameters were used across all sections. The images used for age estimation did not have scale bars attached.

Ageing Procedure

Four laboratories participated in the study: two academic research laboratories and two international governmental fisheries laboratories. The age readers (hereafter referred to as age-readers A-F) had varying levels of expertise as outlined in Table 2.1. Age estimations were made from digital images of the structures in question. A preliminary trial carried out by one age reader (reader A) established that age estimates obtained using images of the structures compared well with estimates obtained from viewing the structures directly under a microscope (coefficient of variation <3%), and age estimation using images was deemed appropriate for the comparison. Clear translucent marks, or groups of more closely packed marks were interpreted as annual bands according to procedures outlined in Megalofonou *et al.* (2003), Santiago and Arrizabalaga (2005) and Fernández (1992) for scales, spines and otoliths, respectively.

Six independent sets of each structure were collated from the available material. In order to compare precision between scales, spines and otoliths from a single fish, all three structures were included in the age assessment exercise. Each set contained images of the relevant structure from 14 randomly selected fish caught in 2005; 7 of Mediterranean origin and 7 of North East Atlantic origin. Images were randomly arranged within each set to ensure that images of spines, scales and otoliths were not presented in the same order to the reader. Sets 1-3 were read by readers B, C and F and Sets 4-6 were read by readers A, D and E. To facilitate a separate comparison of age estimation for two readers that worked closely together, reader A (who had received training in the ageing techniques from reader B) also read sets 1-3. A fourth set of images for each structure was also sent to each reader, this fourth set comprised one of the earlier sets of images rearranged to facilitate the calculation of intra-reader variation. In previous investigations of ageing error in albacore tuna, ages were assigned with knowledge of the length of the fish. In order to examine the potential influence of length data on ageing error, three levels of length information were randomly assigned to the sets of images; no length information, true length information and false length information.

Age Estimation Data Analysis

A combination of statistical and graphical methods was used to compare bias and precision between readers and between structures. The coefficient of variation (CV; calculated as the ratio of the standard deviation to the mean), and percent agreement (the proportion of readings for which paired estimates agree) were used as measures of precision across readers and across structures. ANOVAs and the non-parametric Kruskal Wallis test were used to compare precision (CV) between independent sets of age estimates. Three-way factorial ANOVAs were used to investigate the influence of three factors, stock (two levels; Mediterranean and NE Atlantic), information provided to reader (three levels; length data, no length data and false length data) and reader combination (two levels; A, D & E and B, C & F) on inter-reader precision expressed as the CV of age readings from three readers. Reader combination was included as a random factor, all other factors were fixed. When the interaction terms were non-significant at $P>0.25$, they were pooled with the error term to increase the power of the test for the main effects (Winer *et al.*, 1991). Where significant effects were detected, pair-wise comparisons were carried out using Tukey's post-hoc tests. Age estimates were analysed for each structure separately.

The occurrence of bias (systematic disagreement between readers or structures) in age estimates was visually assessed using age bias plots, where differences between two age-readers or age estimations are plotted as a function of one of the set of ages (Campana *et al.*, 1995). Bias was also tested statistically using linear regression (in Microsoft Excel) and the non-parametric equivalent of a paired t-test, the Wilcoxon's matched-pairs signed ranks test.

Assessing the Degree of Spine Vascularisation

Age assessments in tuna using spines are confounded by the re-absorption or vascularisation of the core region in older animals, which inevitably destroys the annuli from earlier ages (Lee and Yeh, 1993; Megalofonou, 2000; Oritz de Zárate *et al.*, 2007a). The diameter of the first (D_1) and second annulus (D_2) were calculated in fish from the North East Atlantic and Mediterranean sampled in 2005 to 2007 (Figure 2.1) following the procedures in Compean-Jiménez and Bard (1983). ANOVA was used to compare D_1 measurements between age groups; if the second annulus is mistakenly identified as the first annulus in some fish due to vascularisation, and this

source of error is most prevalent in bigger fish, it is expected that D_1 measurements will increase with age. The width of the subsequent growth increment (D_1 - D_2) was also compared between age groups using ANOVA; the rate of growth decreases with age, so if the 2nd annulus if misidentified as the 1st annulus, this will lead to an underestimation of the D_1 - D_2 growth increment and subsequently, the D_1 - D_2 growth increment will be smaller in older fish.

Back Calculation and Accuracy of Growth Estimates

Linear regression analysis was used to determine if spine diameter increased linearly with fish length. Separate analyses of covariance (ANCOVA) were performed for North Atlantic and Mediterranean, comparing total spine diameter between cohorts and between age groups, with fork length included as the covariate in each case; this was to assess cohort and age effects on the spine diameter-fish length relationship. If the factor*covariate interaction term was not significant, it was excluded and the ANCOVA was repeated.

Back calculations of fork length (BCL) were obtained using five back calculation methods; the scale proportional hypotheses (SPH), body proportional hypothesis (BPH), Fraser Lee model, Dahl-Lea model (Francis, 1990) and the age-specific back-calculation method developed by Morita and Matsuishi (2001).

The SPH is based on a linear regression of spine diameter on fish length and is used to back-calculate fish length at a younger age (Francis, 1990)

$$L_i = -\frac{c}{d} + \left(L_c + \frac{c}{d}\right)\frac{D_i}{D_c}$$

Where L_i is the fork length (L_F) at annulus i, D_i the measurement to the ith annual ring, L_c the L_F at time of capture, D_c is the spine diameter at capture, and c and d are the intercept and slope, respectively, from the regression of spine diameter on L_F.

The BPH is based on the linear regression of the fish length on spine diameter (Francis, 1990)

$$L_i = \frac{a + bD_i}{a + bD_c} L_c$$

Where a and b are the intercept and slope, respectively, from the regression of L_F on spine diameter

The Fraser-Lee method is a linear, direct proportional back calculation method (Fraser, 1916 & Lee, 1920) cited in (Francis, 1990), which back-calculates length for individual fish as

$$L_i = a + (L_c - a)\frac{D_i}{D_c}$$

where a is a correction factor. The correction factor a proposed by Fraser (1916) in Francis (1990) represents the length of the fish at the time of bony structure formation. The correction factor can be derived mathematically from the y-intercept of a linear regression of body length on bony structure radius (Lee 1920; Francis, 1990).

The direct proportional or Dahl–Lea model (Dahl 1907; Lea 1910 cited in Francis (1990) has a zero intercept, and thus reduces the Fraser–Lee model to a linear model passing through the origin.

$$L_i = \left(\frac{D_i}{D_c}\right)L_c$$

The SPH, BPH, Fraser-Lee and Dahl-Lea models all assume constant proportionality between structure size and fish length. The back-calculation model of Morita and Matsuishi (2001) incorporates age effects onto the structure size-fish length relationship. This model is based on multiple regressions of structure size and fish age on fish length.

Fish lengths are back calculated using the equation

$$L_i = -\frac{x}{y} + \left(L_c + \frac{x}{y} + \frac{z}{y}T\right)\frac{D_i}{D_c} - \frac{z}{y}t$$

Where t is fish age at time of capture T, and x, y and z are constants derived from the multiple regression of spine diameter on fish length and age.

$$D_c = x + yL_c + zt$$

Separate univariate ANOVA's for the two regions (North Atlantic and Mediterranean) were used to determine the influence of age group on the back calculated length at age 1 (BCL_1). The BCL_1 estimates derived from each of the five back-calculation methods were compared to each other using paired t-tests in Minitab 15® for windows. The length at estimated age results for ages 1-3 from the back calculation methods were compared to hypothetical lengths at age used by ICCAT from the Von Bertalanffy growth parameters (ICCAT, 2003).

The (von Bertalanffy, 1938) growth curve is

$$L_t = L_\infty \left\{ 1 - e^{\left[K^{(y^{-1})} (t - t_0^{(y)}) \right]} \right\}$$

Where L_∞ is the length at maximum age, K = growth per year and t_0 is the theoretical length at age 0. As reported growth rates for albacore vary between years and geographic areas (Santiago and Arrizabalaga, 2005), the back calculation lengths were also compared to the Von Bertalanffy (1938) growth curve derived from length at age and catch data for the samples used in the age assessment within this study from 2005 to 2007 where K = 0.15, L_∞ = 120.44 and t_0 = -2.01.

2.4. Results

The length of the fish used in the study ranged from 50.0 cm to 79.3 cm fork length with a mean length of 62.5 cm. The assigned ages ranged from 0-5 years. Age estimates indicated that the samples were dominated by two and three year old fish. The mean lengths of fish used in the comparative age assessment, within each age class assigned by reader A, who aged all structures, are shown in Table 2.2.

Inter-Reader Precision

The comparison of reader B (experienced reader) with reader A (inexperienced reader trained by reader B) yielded moderate levels of precision (Table 2.3). For the Mediterranean albacore, the mean CV was consistently below 9% and percent agreement was above 70% for all structures; the greatest between reader agreements was achieved using otoliths. For the NE Atlantic albacore, good precision was obtained using spines (CV, 5%; percent agreement, 86%), while the level of precision achieved using otoliths was low (CV, 20%; percent agreement, 38.1%). Kruskal Wallis tests confirmed that the mean CV of otolith age estimates from readers A and B was significantly lower for the Mediterranean fish than the NE Atlantic fish ($P<0.01$), indicating better inter-reader precision for the Mediterranean fish. No difference in precision between NE Atlantic and Mediterranean age readings was detected for the scales and spines ($P>0.05$). For readers A and B, there was no significant difference in mean CV between sets read with length data, with no length data and with false length data for the scales, spines and otoliths for both areas ($P<0.05$). Therefore, the inclusion of length data did not influence the inter-reader precision of age estimates for these readers. The wider comparison of readers from across laboratories showed poor precision (Table 2.3). The mean CV was above 20% for all structures from both Mediterranean and NE Atlantic albacore and percent agreement ranged from 45% down to 25%.

In the case of the age estimates from otoliths, the three-way ANOVA (Table 2.4) revealed that inter-reader precision was influenced by the combination of readers involved in the comparison, the length information provided to the reader and the source of the otoliths (Mediterranean or NE Atlantic). Tukey post-hoc tests showed that the mean CV was significantly lower between readers B, C and F compared with

readers A, D and E ($P<0.05$), indicating greater inter-reader precision in the first group of readers. Better inter-reader precision was achieved with the otoliths from Mediterranean albacore when compared to the NE Atlantic albacore, with the age estimates for the former group showing a significantly lower mean CV (Tukey post-hoc test, $P<0.05$). The influence of length information on inter-reader precision was difficult to interpret, with significantly lower CV's recorded for the sets that were read with false length information compared to those that were read with true length information (Tukey post-hoc test, $P<0.05$). For the scales, inter-reader precision was influenced by just one of the main effects in the model; stock (Table 2.4). Better inter-reader precision was achieved with scales from Mediterranean albacore when compared to the NE Atlantic albacore, with the age estimates for the former group showing a significantly lower mean CV (Tukey post-hoc test, $P<0.05$). With respect to age estimates derived from spines, both stock and length information influenced inter-reader precision (Table 2.4). Better inter-reader precision was achieved with spines from Mediterranean albacore when compared to the NE Atlantic albacore, with the age estimates for the former group showing a significantly lower mean CV (Tukey post-hoc test, $P<0.05$). The mean CV of age estimates obtained with no length information was significantly higher than the mean CV of estimates made with the true length of the fish provided (Tukey post-hoc test, $P<0.05$), indicating that the provision of length information may artificially reduce inter-reader precision for spine age estimates.

Inter-Reader Bias

Linear regression analysis found no significant systematic differences in age estimates between readers A and B for otoliths, scales or spines. However, significant bias was detected for all other reader combinations and for all structures (Table 2.5). For the majority of reader pairings, the slope of the relationship between the two sets of age estimates was significantly different from one, indicating inconsistencies in the estimates of one reader. In addition, the intercept was significantly different than zero for most of the linear regressions. This signifies over-estimation or under-estimation of ages by one reader relative to the other. Relative bias is also apparent in the age-bias plots shown in Figure 2.2. It is important to note that as the absolute age of the fish is not known the actual direction of the bias can not be determined and readers'

estimations are only assessed relative to each other. Within the first group of three readers (A, D, E), reader D showed a tendency to underestimate age relative to the other readers. The age estimates for reader E tended to be higher than the corresponding ages supplied by readers A and D, with the exception of the otolith ages which reader E appeared to underestimate relative to reader A. Within the second group of readers (B, C, F), the trend suggests over-estimation of age by reader C and underestimation by reader F relative to the other readers. Comparison of reader A with reader B indicates a slight tendency for reader A to overestimate age relative to reader B. Wilcoxon one-sample signed ranks tests showed that for most comparisons; the systematic inter-reader bias apparent in the age bias plots was statistically significant (Table 2.5).

Bias and Precision between Structures

Considerable inconsistencies were detected in the age estimates derived from two structures in the same fish (Table 2.6) for both Mediterranean and NE Atlantic albacore. The mean CV of the paired comparisons between structures (all readers combined) ranged from 16 to 25%, mean percent agreement ranged from 39 to 52%. The most experienced reader, B, achieved high levels of precision between structures for the Mediterranean fish but relatively low precision for fish from the NE Atlantic. The non-parametric Wilcoxon's matched-pairs signed rank test showed significant systematic bias in the age estimates obtained from one structure relative to another (Table 2.7). The occurrence and direction of the bias was not consistent between readers. Reader A showed no tendency to over- or underestimate age from one structure relative to another while reader E showed some degree of bias for all structures. Higher age estimates were derived from scales compared to spines for three readers. The bias between otoliths and scales and between otoliths and spines was not consistent between readers.

Within Reader Precision

Mean CV's of repeat readings ranged from 0% in readers A and B for scales and spines, respectively, to 43.2% in spine age estimations of reader F. The lowest percentage agreement obtained (7%) was from repeat readings of otoliths by age

reader E (Table 2.8). Analysis of variance was used to determine if there were significant differences in the precision achieved between different readers, or between structures. Significant differences were present in the repeated readings between age readers, and there was an interaction between reader and structure. Tukey's posthoc test showed that the precision of age reader F within all structures was significantly lower compared to the other age readers ($P<0.05$). Age reader E has lower levels of precision in repeat age estimations in otoliths ($P<0.05$). All other comparisons between age readers within structures showed no significant differences.

Vascularisation in Dorsal Spines

The first annulus in the dorsal spine (D_1) was measured in 650 albacores, 31 were removed from analysis as these were present in small numbers in either the age class or cohort. D_1 measurements are summarised in Table 2.9. ANOVA with Tukey's post hoc test shows that for the NE Atlantic fish, D_1 is significantly smaller in 2 year old fish compared to 3, 4 and 5 year old fish and in 3 and 4 year old fish compared to 5 year old fish (Table 2.10). However, comparison of the second growth increment (D_1 - D_2) between age groups found no evidence of a decrease with age (Table 2.11); in fact the D_1 - D_2 growth increment was smaller in 2 year old fish compared to 5 year old fish. This suggests that the observed differences in annuli measurements between age groups are not due to obscuring of the 1st annuli in older fish. For the Mediterranean fish, there was no evidence that the 1st annulus was obscured in older fish, as D_1 did not increase with age but was significantly larger in 4 year old fish compared to 5 year old fish (Table 2.10). Comparison of the D_1 - D_2 measurement between age groups found that the growth increment was larger in 3 year old fish compared to 4 year old fish (Table 2.11).

Back-Calculation and Growth Parameters

A total of 650 fish sampled between 2005 and 2007 were aged using spine structures by age reader A, 501 from the North East Atlantic Ocean and 149 from the Mediterranean Sea. A strong linear relationship was observed between the total diameter of the spine sections and fork length of fish in both regions (Figure 2.3) justifying the back-calculation of size from spine measurements.

The relationship of the total spine diameter (D) on fish length within cohort (2001 to 2004) is shown in Figure 2.4. The relationship appears to vary between cohorts. ANCOVA confirmed that there were significant differences in the slopes (cohort*length interaction term) and intercepts (cohort term) of the spine diameter-fish size relationship between cohorts in both the NE Atlantic and the Mediterranean fish ($P<0.05$; Table 2.12). This indicates that the diameter of the spine at a given length and the rate at which spine diameter changes with fish length varies between cohorts.

ANCOVA was also used to compare the spine diameter-fish size relationship between age-groups. There were no significant differences in the slopes of the spine diameter-fish size relationship between age-groups for fish from both regions ($P>0.05$; Table 2.12). After the interaction terms were removed, significant differences were observed in the intercepts of the spine diameter -fish length relationship, i.e. at any given length there is a significant difference in the size of the spine between the age groups.

Paired t-tests comparing back-calculated lengths at age 1 estimates (BCL_1) showed that estimates derived from the BPH and Fraser-Lee models were not significantly different ($P>0.05$) to each other, however all other paired comparisons showed significant differences ($P<0.05$). ANOVA with age group as a fixed factor showed no significant differences in estimated BCL_1 within the age groups for the SPH, Morita and Matsuishi (2001) and Dahl-Lea models in the NE Atlantic ($P>0.05$). However significant differences in BCL_1 ($P<0.05$) were evident between age groups (two to five years old) for the BPH and Fraser-Lee models; post-hoc tests indicated that the BCL_1 for two year old fish was significantly less than for all other age classes (three to five). No significant differences in BCL_1 between the age groups (three to six) were evident in fish from the Mediterranean Sea for any of the models.

Estimated mean lengths at age derived form the five back calculation methods are shown in Table 2.13. These values were compared graphically with the ICCAT estimated length at ages calculated from the Von Bertalanffy (1938) growth parameters presented in ICCAT (2003) between the ages of 1 and 3 (Figure 2.5). The intercept on the y-axis was set as size at hatch from Piccinetti *et al.* (1973) of 7.4mm. This graph shows that the BCL_1 estimates derived using the SPH, Dahl-Lea and Morita and Matsuishi (2001) models are consistent with the ICCAT estimated length at age, however BCL_1 estimates derived using the BPH and Fraser-Lee models show

an overestimation of length at age 1 relative to the ICCAT growth curve. All BCL_2 and BCL_3 estimates from were less than those expected from the ICCAT Von Bertalanffy (1938) growth curve (Figure 2.5).

2.5. Discussion

Ageing Precision

The fish used in this study were restricted to juveniles <80cm and age estimates indicate that 2 and 3 were the dominant age classes. The mean lengths of age classes 3 and 4 were lower than those reported in studies of age and growth of Mediterranean (Megalofonou, 2000) and North Atlantic albacore (Santiago and Arrizabalaga, 2005). As the younger age classes were not affected, it is likely that this discrepancy reflects the restricted length distribution of the samples rather than a major difference in the interpretation of annuli.

The level of precision observed in the comparison of spine age estimates of readers B (experienced reader) and A (inexperienced reader trained by reader B) is similar to previous reports of inter-reader precision within a laboratory (Oritz de Zárate *et al.*, 2005). This corroborates the assertion that reasonable precision can be achieved with albacore spine sections when the ageing methodology is standardised between readers. The precision achieved for the scales and otoliths of Mediterranean fish was also acceptable and supports the suitability of these structures for estimating age in this stock. The low precision observed in the otolith age estimates for the NE Atlantic fish indicates that the interpretation of otolith annuli may be prone to a degree of subjectivity for this stock, even with cross-calibration of methodologies.

Although acceptable precision was achieved by readers A and B, poor precision was observed in all other comparisons between readers, especially for otoliths. In addition, most inter-reader comparisons (including readers A and B) showed some degree of bias with one reader over- or underestimating age relative to the other. There were also considerable inconsistencies and systematic differences between age estimates derived using different structures from the same fish. In some cases the choice of one ageing structure over another could cause bias in the estimation of length at age. In particular, scales appear to produce lower estimates of age compared to spines. The results emphasize the need for much greater standardisation of ageing methodologies across laboratories through further exchange of material and consultation between readers. In particular, uncertainty surrounding the interpretation of multiple banding needs to be resolved (Oritz de Zárate *et al.*, 2007a). Inter-calibration of the ageing methodologies used for each structure may

help to reduce variability between estimates derived from otoliths, scales and spines and ensure that the annual bands identified in one structure correspond to those identified in another. This is especially pertinent where features in one structure are used to validate annual marks in another. Inter-calibration could increase precision between laboratories to the levels observed within laboratories as reported above. This is critical to ensure that age information from different jurisdictions is reliable and comparable and that spatial and temporal variations in growth rates are not an artefact of the ageing methodologies employed.

Higher precision was achieved using material from Mediterranean albacore than from NE Atlantic albacore. The apparent difference in increment clarity may reflect a real stock-related difference in the conditions experienced across migration pathways that influence the deposition of annual growth bands in the calcareous structures. This highlights the importance of verifying ageing procedures for each stock and for putative components within stocks, which may have experienced different environmental conditions. Further comparison of material from each area across a wider range of length classes is needed to confirm if annual growth bands are more variable and more difficult to interpret in NE Atlantic albacore.

Vascularisation in Spines

Dorsal spines are the favoured ageing structure in albacore tuna (ICCAT, 2003). However these structures are metabolically active and the central region of the core is re-absorbed as the fish gets older (Compean-Jiménez and Bard, 1983; Megalofonou, 2000; Oritz de Zárate *et al.*, 2007a). This vascularisation, which is also observed in other tuna species, can hinder both precise and accurate age assessments of albacore as the exact location of the first annual increment may not be known.

In this study the size of the first annulus and the width of subsequent growth increments were compared between age groups to assess the potential impact of vascularisation on the age estimates. It was expected that if vascularisation led to underestimation of age in older fish, this would result in an increase in the size of the first annulus with age and a decrease in the width of the subsequent growth increment. For the NE Atlantic fish, although the size of the first annulus did increase with age, the D_1-D_2 growth increment was not smaller in older fish. For the Mediterranean fish, the growth increment was slightly larger in 3 year old fish compared to 4 year olds,

but there was no corresponding difference in the diameter of the first observed annulus. Therefore it is concluded that in this study vascularisation did not have a significant effect on the age estimations of fish from 2 to 5 years old. To further assess the effect of vascularisation on age estimations a large range of age classes are required, i.e. fish older than 5 years. An alternative explanation for the observed increase in the size of the first annulus with age in North Atlantic albacore is length-dependant mortality, with enhanced survival of fast growing fish leading to disproportionate representation of these fish in the older age groups (Ricker, 1969).

Back Calculation and Growth

Back calculated lengths at age 1 ranged from 35 to 60cm, with median values at approximately 47cm, which is consistent with previous studies (Santiago, 1993; Lee and Yeh, 2007). The relationship between spine diameter and fish size was linear, justifying the back-calculation of fish size from measurements of spine annuli. ANCOVA showed that the relationship between dorsal spine diameter and fish length was not consistent across cohorts and age groups. Otoliths from slow growing fish are often larger than otoliths from fast-growing fish of the same size (Reznick *et al.*, 1989; Secor *et al.*, 1989; Campana, 1990) as otoliths can continue to grow in the absence of somatic growth. Although it is not known if the same phenomenon occurs in dorsal spines, the observed differences in the spine diameter fish size relationship may reflect cohort- and age-specific variation in growth rates in both North Atlantic and Mediterranean albacore. Further confirmation of the effect of growth rates on the spine-diameter fish size relationship is needed. Nonetheless, the age- and cohort-specific differences in the spine diameter fish size relationship suggest that a back-calculation that incorporates this effect Morita and Matsuishi (2001) would be the most appropriate.

Significant differences were observed between age classes (age 2 versus all older fish) in the NE Atlantic samples using the BPH and Fraser-Lee models. Previous studies have shown that the Fraser-Lee method may suffer from inconsistencies in back calculations where mean length at age may differ from actual lengths at age (Campana, 1990). In this study BCL_1 calculated using the Fraser-Lee method were overestimated compared to the theoretical ICCAT length at age 1 (Figure 2.5), the BPH model also showed an over-estimation at BCL_1. The other

three models returned similar BCL_1 to the ICCAT model. All methods indicated an underestimation of mean length at age for ages 2 and 3 relative to the ICCAT model. However, when the BCL's are compared to the Von Bertalanffy (1938) parameters estimated from the length at age data for all albacores used in this study the problem of under-estimation is removed. It therefore appears that the differences in BCL's compared to the ICCAT predicted lengths at ages are a sampling artefact.

This study deals only with precision and relative bias and not with accuracy of age estimates. The development and standardisation of ageing protocols should be supported by validation studies using daily increment counts or tag recapture studies to ensure that age estimates reflect the absolute age of the fish and are accurate as well as precise. Further inter-laboratory collaborations are required to improve precision between a large numbers of age readers who contribute towards management information. Although D_1 appeared to be consistently identified in spines of fish up to 5 years old, the differences observed in sizes may be attributed to discrepancies in interpretation or real changes in growth rates between cohorts. Additional studies on differences in growth rates, through mark-recapture studies using oxytetracycline, may provide additional information on cohort specific growth. As an age/cohort effect is evident in spine diameter is seems that the Morita and Matsuishi (2001) back calculation model is most appropriate for use in future investigations where the length of albacores in an earlier age class is determined, as this model incorporates age specific effects. As cohort specific differences were observed in spine diameter and back calculation it is evident that more information is required on the differences in yearly growth rates of the albacore cohorts as they progress through the fishery. This information would aid management of albacore in the North Atlantic and Mediterranean Sea.

Table 2.1. Experience of readers with estimating the age of fish from otoliths, scales and dorsal fin ray spines. The ordinal values for experience are 0 = no experience, 1 = experience with other species, and 2 = experience with albacore tuna.

	Otoliths	*Scales*	*Spines*
A	0	0	0
B	2	2	2
C	0	0	0
D	1	0	0
E	2	0	1
F	1	0	1

Table 2.2. Mean length of each age class assigned by reader A for otoliths, scales and spines ± 95% confidence intervals. The numbers in each class are shown in brackets.

	NE Atlantic			Mediterranean		
Age Class	*Otoliths*	*Scales*	*Spines*	*Otoliths*	*Scales*	*Spines*
1		54.3 ± 8.2 (5)	51.2 ± 1.0 (4)			
2	55.7 ± 4.7 (11)	55.8 ± 2.5 (7)	62.0 ± 4.1 (15)	62.2 ± 0.82 (12)	62.4 ± 0.64 (25)	62.4 ± 0.64 (20)
3	62.1 ± 2.3 (24)	62.2 ± 2.4 (15)	61.6 ± 3.6 (16)	63.0 ± 1.1 (22)	63.4 ± 1.3 (15)	63.1 ± 1.3 (18)
4	71.6 ± 7.4 (7)	67.5 ± 4.6 (15)	69.9 ± 6.9 (4)	65.6 ± 5.9 (4)	66.8 ± 2.6 (2)	64.8 ± 6.3 (4)

Table 2.3. Inter-reader precision of age-estimates for otoliths, scales and spines from North East Atlantic (NEAtl) and Mediterranean (Med) albacore. Standard errors are shown in brackets.

	Readers A and B					
	Otoliths		*Scales*		*Spines*	
	NEAtl	Med	NEAtl	Med	NEAtl	Med
% Agreement (averaged across comparisons)	38.1	90.5	61.9	81.0	85.7	71.4
Mean CV (%)	19.8 (± 3.8)	2.7 (± 1.9)	10.0 (± 2.9)	5.4 (± 2.5)	4.9 (± 2.8)	8.1 (± 2.9)
N	21	21	21	21	21	21
	All readers					
	Otolith		*Scales*		*Spines*	
	NEAtl	Med	NEAlt	Med	NEAlt	Med
% Agreement (averaged across comparisons)	28.2 (± 5.7)	24.7 (± 4.3)	35.6 (± 2.0)	45.3 (± 2.2)	25.4 (± 1.3)	38.7 (± 2.6)
Mean CV (%)	39.6 (± 2.6)	31.8 (± 2.1)	28.4 (± 2.9)	22.9 (± 2.5)	26.8 (± 2.5)	21.2 (± 2.9)
N	40	42	37	41	38	41

Table 2.4. Three-way ANOVA summary table showing the influence of inter-reader precision (%CV) achieved from age-estimations for North East Atlantic and Mediterranean albacore.

Source of variation	d.f.	MS	F-ratio	p
Otoliths				
Stock (S)	1	0.13	6.6	0.012*
Length information (L)	1	0.09	4.5	0.014*
Reader combination (R)	2	0.09	4.8	0.032*
S*L				>0.25
S*R				>0.25
L*R				>0.25
S*L*R				>0.25
Error	79	0.019		
Scales				
Stock (S)	1	0.10	4.19	0.044*
Length information (L)	2	0.87	1.24	0.45
Reader combination (R)	1	0.0043	0.06	0.83
S*L	2	0.066		0.072
S*R				>0.25
L*R	2	0.070		0.062
S*L*R				>0.25
Error	75	0.024		
Spines				
Stock (S)	1	0.070	4.29	0.041*
Length information (L)	2	0.056	0.01	0.036*
Reader combination (R)	1	0.00014	3.47	0.926
S*L				>0.25
S*R				>0.25
L*R				>0.25
S*L*R				>0.25
Error	83	1.29		

Table 2.5. Results of linear regression analysis of Inter-reader bias.

Statistic	Reader A vs. Reader B			Reader A vs. Reader D		
	Otolith	*Scale*	*Spine*	*Otolith*	*Scale*	*Spine*
Slope	1.149±0.45	1.003±0.25	1.122±0.37	0.214±0.29	0.729±0.43	0.376±0.34
p value	0.000	0.000	0.000	0.139	0.001	0.033
Intercept	-0.050±0.88	0.232±0.55	-0.041±0.73	2.669±0.60	1.538±0.91	2.192±0.83
p value	0.910	0.397	0.911	<0.001	0.002	<0.001
Test of Slope =1	p>0.05	p>0.05	p>0.05	p<0.05	p>0.05	p<0.05
Wilcoxon test	p=0.002 A>B	p=0.006 A>B	p=0.004 A>B	p<0.001 A>D	p<0.001 A>D	p<0.001 A>D
	Reader B vs. Reader C			**Reader B vs. Reader F**		
	Otolith	*Scale*	*Spine*	*Otolith*	*Scale*	*Spine*
Slope	0.697±0.25	0.554±0.23	0.246±0.13	0.391±0.40	0.278±0.29	0.323±0.14
p value	<0.001	<0.001	<0.001	0.056	0.058	<0.001
Intercept	-0.032±0.83	0.682±0.60	1.249±0.38	1.609±0.66	1.604±0.47	1.266±0.30
p value	0.939	0.028	<0.001	<0.001	<0.001	<0.001
Test of Slope =1	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05
Wilcoxon test	p<0.001 C>B	p<0.001 C>B	p<0.001 C>B	p<0.001 B>F	p<0.001 B>F	p>0.05

Reader A vs. Reader E		
Otolith	*Scale*	*Spine*
0.284±0.13	0.497±0.22	0.250±0.20
0.000	0.000	0.017
2.429±0.34	1.556±0.71	2.200±0.73
<0.001	<0.001	<0.001
p<0.05	p<0.05	p<0.05
p<0.001 A>E	p>0.05	p=0.02 A<E

Reader D vs. Reader E		
Otolith	*Scale*	*Spine*
0.100±0.17	0.003±0.18	0.197±0.17
0.236	0.785	0.022
1.760±0.44	1.998±0.59	1.684±0.61
<0.001	<0.001	<0.001
p<0.05	p<0.05	p<0.05
p>0.05	p<0.001 D<E	p<0.001 D<E

Reader C vs. Reader F		
Otolith	*Scale*	*Spine*
0.490±0.36	0.361±0.35	0.506±0.34
0.009	0.044	0.005
2.4546±0.59	1.905±0.58	1.790±0.75
<0.001	<0.001	<0.001
p<0.05	p<0.05	p<0.05
p<0.001 C>F	p<0.001 C>F	p<0.001 C>F

Table 2.6. Precision of age-estimates between structures for North East Atlantic (NEAtl) and Mediterranean (Med) albacore.

	Otoliths vs. scales		*Otoliths vs. spines*		*Scales vs. spines*	
	NEAtl	Med	NEAtl	Med	NEAtl	Med
% Agreement						
Reader A	47.6	47.6	52.4	57.1	42.9	52.4
Reader B	42.9	95.2	61.9	85.7	57.1	95.2
Reader C	36.8	40.0	44.4	55.0	41.2	42.1
Reader D	50.0	52.4	35.3	37.0	61.1	38.7
Reader E	9.5	14.3	15.8	28.6	33.3	21.1
Reader F	47.1	61.1	45.0	42.9	44.4	42.1
Mean % agreement	39.0	51.8	42.5	51.1	41.2	45.1
CV (%)						
Reader A	13.9	14.0	12.8	10.6	13.1	15.3
Reader B	21.2	5.39	17.1	4.0	18.0	1.3
Reader C	21.1	19.0	17.1	13.0	21.7	17.4
Reader D	16.8	19.1	24.1	18.6	15.5	13.9
Reader E	41.9	30.7	41.6	27.5	18.8	26.4
Reader F	36.0	18.3	25.0	23.8	41.9	30.9
Mean CV (%)	25.2	17.8	22.9	16.3	21.5	17.5
N	21	21	21	21	21	21

Table 2.7. Results from Wilcoxon's paired tests used to detect systematic bias in age readings between structures for each reader. * indicates statistical significance at the alpha = 0.05 level, ** indicates statistical significance after Bonferroni correction for multiple comparisons, ns indicates non-significance, ot= otolith, sc=scale and sp = spine.

	Otoliths vs. scales		*Otoliths vs. spines*		*Scales vs. spines*	
	p value	Bias	p value	Bias	p value	Bias
Reader A	ns	-	ns	-	ns	-
Reader B	ns	-	0.03*	ot>sp	ns	-
Reader C	0.0002**	ot>sc	ns	-	ns	-
Reader D	ns	-	ns	-	0.02*	sp>sc
Reader E	0.03*	sc>ot	0.0008**	sp>ot	0.03*	sp>sc
Reader F	ns		0.0004**	sp>ot	0.003**	sp>sc

Table 2.8. Mean CV and percentage agreement of repeated age estimations for each structure by readers **A** – F.

Reader		*Otolith*	*Scale*	*Spine*	*Average*
A	Mean CV (%)	4.0	0.0	2.9	2.3
	% Agreement	85.7	100.0	85.7	90.47
B	Mean CV (%)	10.8	10.4	0.0	7.07
	% Agreement	71.4	78.5	100.0	83.30
C	Mean CV (%)	11.5	13.3	10.2	11.67
	% Agreement	42.9	64.3	69.2	58.80
D	Mean CV (%)	29.0	2.2	7.7	12.97
	% Agreement	33.3	92.3	72.7	66.10
E	Mean CV (%)	32.8	13.6	5.1	17.17
	% Agreement	7.1	50.0	71.4	42.84
F	Mean CV (%)	13.5	37.7	43.2	31.47
	% Agreement	71.4	60.0	33.3	54.91

Table 2.11. Results of ANOVA comparing the second growth increment ($D_1 - D_2$) between age groups (ages 2 to 5) for the North East Atlantic and Mediterranean samples.

	Mean-square	DF	F	P	r2	Tukey's Post Hoc Test
NE Atlantic	413507	3	3.84	0.010	3.20	2<5
Mediterranean	100423	2	3.51	0.033	4.80	3>4

Table 2.12. Results of ANCOVA comparing total spine diameter (D) between age groups and cohorts with fork length (L_F) included as the covariate for North East Atlantic and Mediterranean albacores.

	Mean-square	DF	F	P	r^2
NE Atlantic Spine Diameter vs. Age Group					
Age Group	66208994	3	6.19	0.000	89.77
L_F (mm)	17618983	1	337.51	0.000	
Age Group*L_F (mm)	28037	3	0.54	0.658	
NE Atlantic Spine Diameter vs. Cohort					
Cohort	37792658	3	4.11	0.007	90.03
L_F (mm)	102970234	1	1073.66	0.000	
Cohort*L_F (mm)	174323	3	3.4	0.018	
Mediterranean Spine Diameter vs. Age Group					
Age Group	2572379	2	3.04	0.051	74.04
L_F (mm)	6898712	1	255.40	0.000	
Age Group*L_F (mm)	32829	2	1.07	0.345	
Mediterranean Spine Diameter vs. Cohort					
Cohort	83110	3	5.18	0.002	77.38
L_F (mm)	249329	1	28.14	0.000	
Cohort*L_F (mm)	3975479	3	4.99	0.003	

Table 2.13. Length at estimated ages calculated using the SPH, BPH, Morita and Matsuishi (2001), Fraser-Lee and Dahl-Lea back calculation methods for North East Atlantic and Mediterranean fish caught between 2005 and 2007. Also shown is the estimated length at age from Von Bertalanffy growth parameters used in ICCAT albacore stock assessments. Lengths shown are in centimetres.

Estimated Age	N		SPH Model Mean BCL		BPH Model Mean BCL		Morita and Matsuishi, (2001) Mean BCL		Fraser-Lee Mean BCL		Dahl-Lea Mean BCL		ICCAT estimated Length at Ages
	North Atlantic Ocean	Mediterranean Sea	North Atlantic Ocean	Mediterranean Sea	North Atlantic Ocean	Mediterranean Sea	North Atlantic Ocean	Mediterranean Sea	North Atlantic Ocean	Mediterranean Sea	North Atlantic Ocean	Mediterranean Sea	
1	501	149	44.4	45.5	50.3	51.7	44	45.5	50.2	51.7	43.9	44.5	44.16
2	376	148	54.9	55.9	58.6	59.1	54.5	56.3	58.6	59.1	54.5	55.3	60.72
3	209	92	63.5	61.9	66.2	64.1	63	62.5	66.2	64	63.2	61.6	73.87

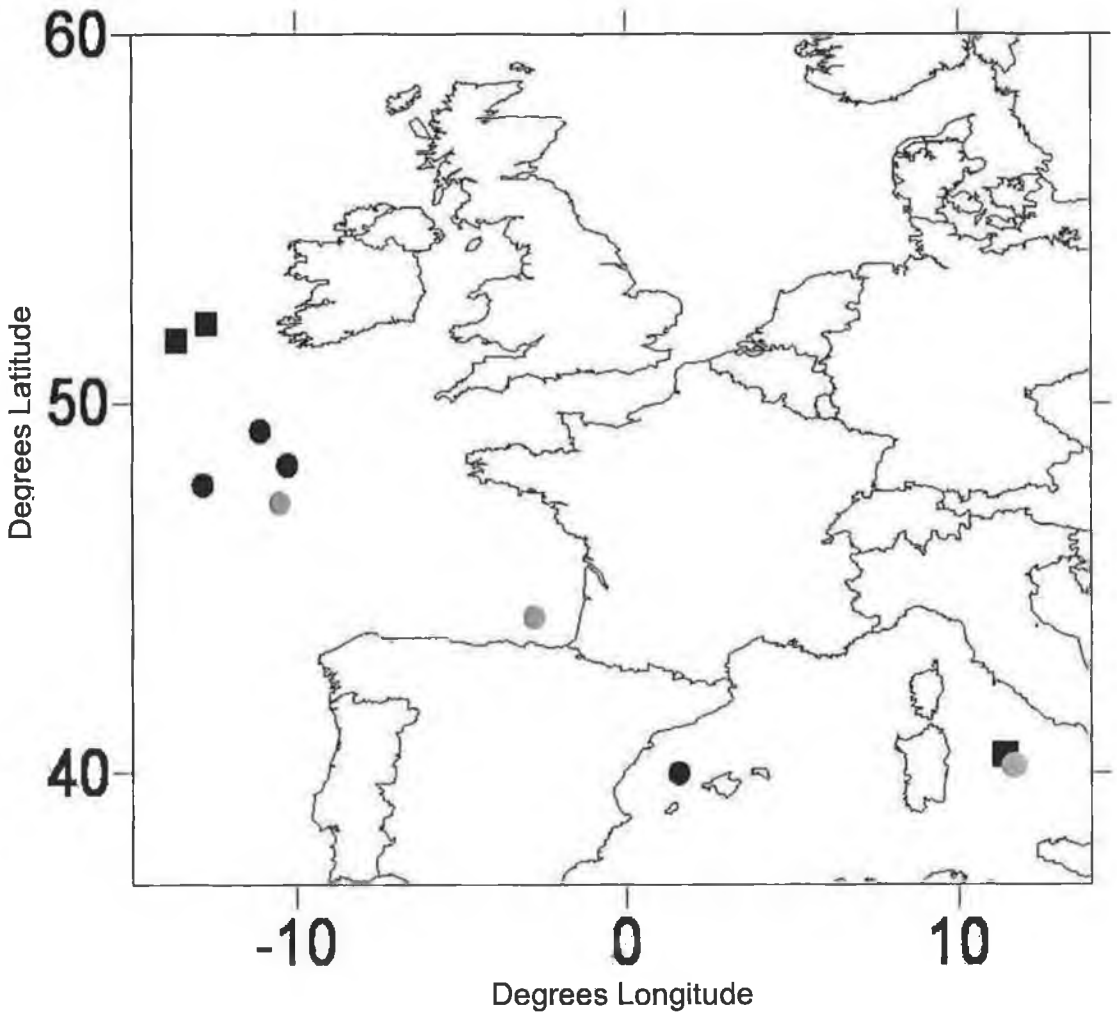

Figure 2.1. Geographic locations of sample collection in 2005 (●), 2006 (●) and 2007(■).

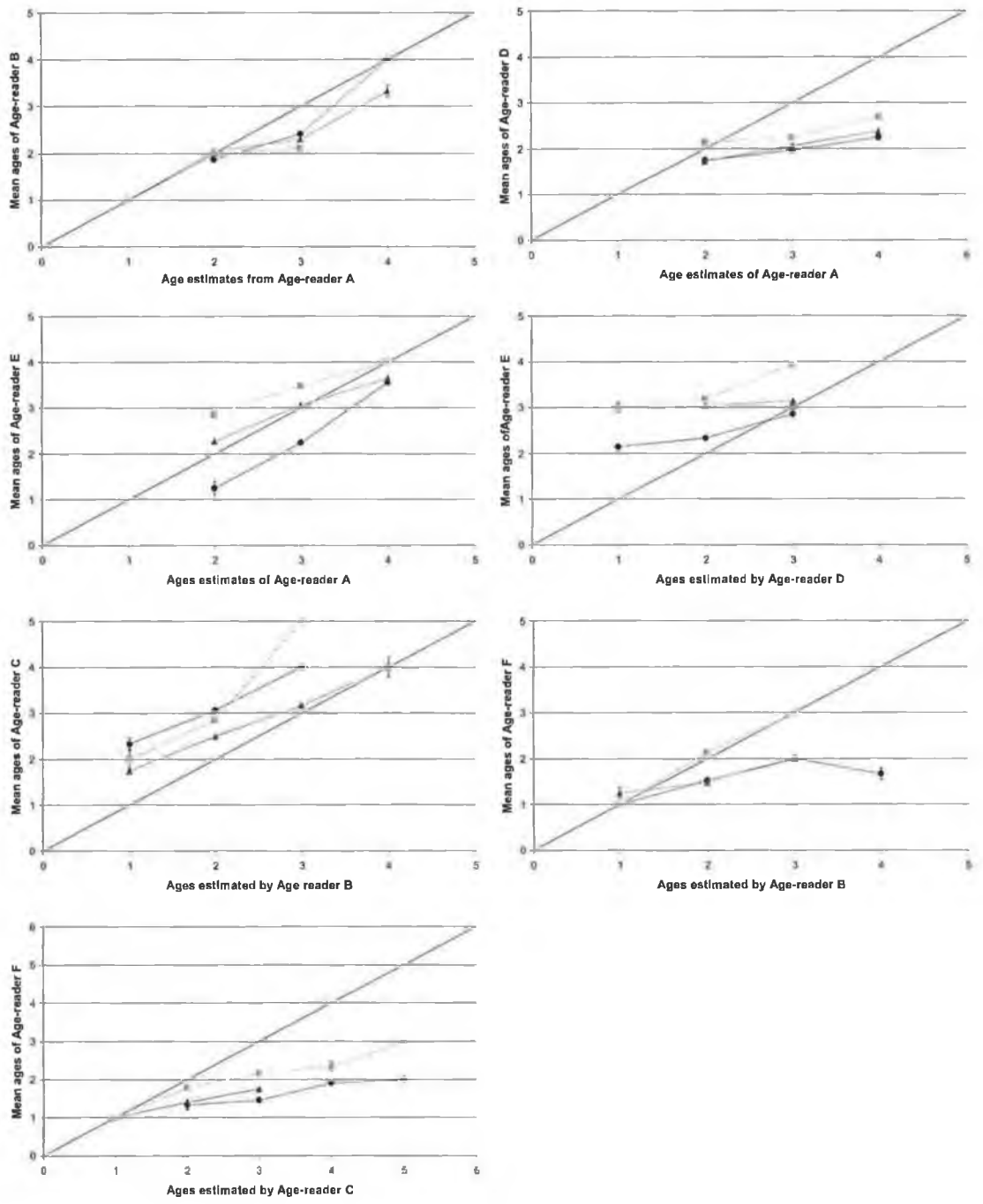

Figure 2.2. Age bias plots for each of the seven paired age comparisons for the three structures; otoliths (●), scales (▲) and spines (■). Error bars represent the 95% confidence intervals about the average age assigned by one age-reader for all fish assigned a certain age by another age-reader. The 1:1, or zero difference line (solid line) is indicated.

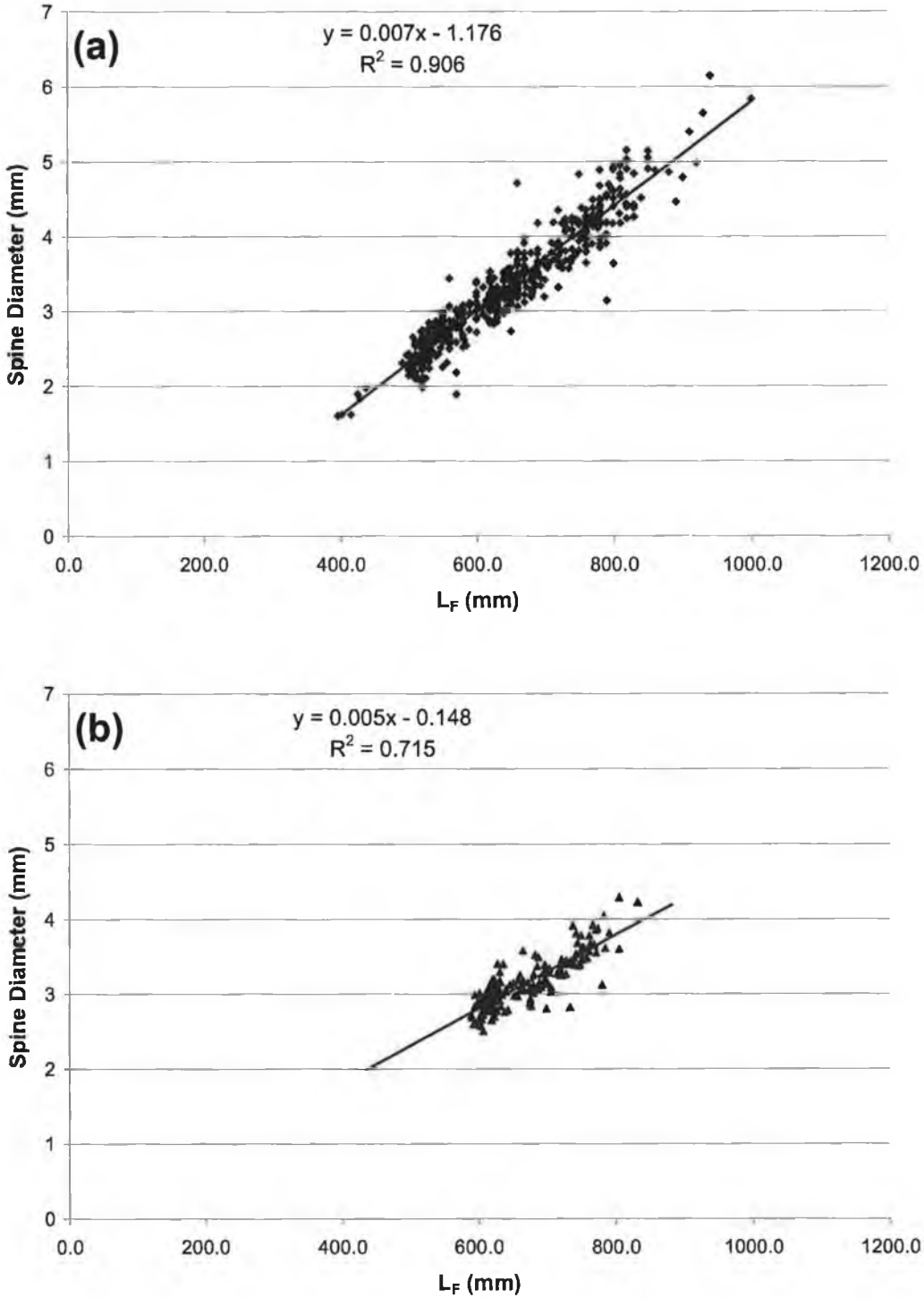

Figure 2.3. Relationships between fish length (L_F) and total spine section diameter. Regression lines are shown for (a) the NE Atlantic Ocean and (b) the Mediterranean Sea.

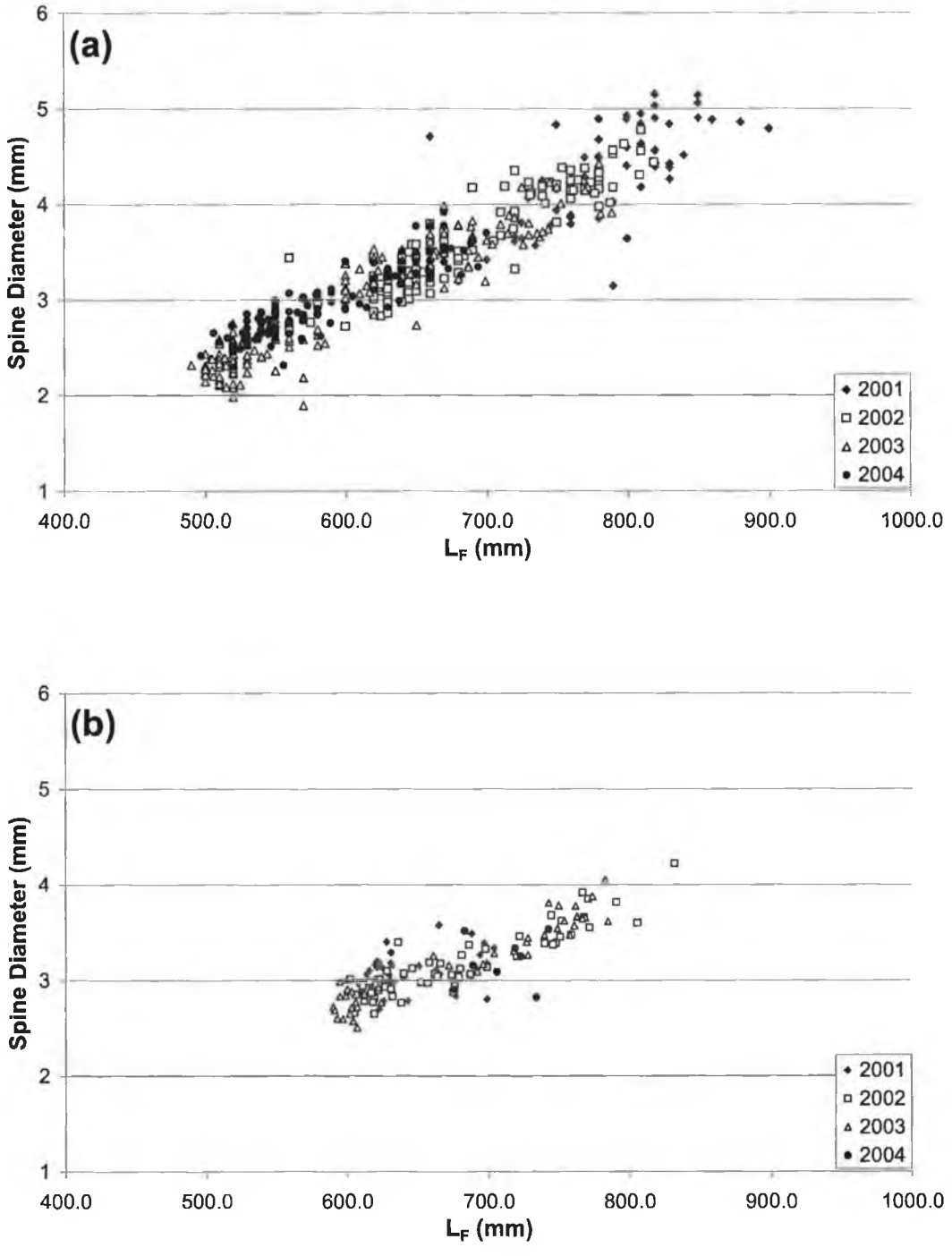

Figure 2.4. Relationships between fish length (L_F) and total spine section diameter between cohorts 2001 to 2004 in (a) the NE Atlantic Ocean and (b) the Mediterranean Sea.

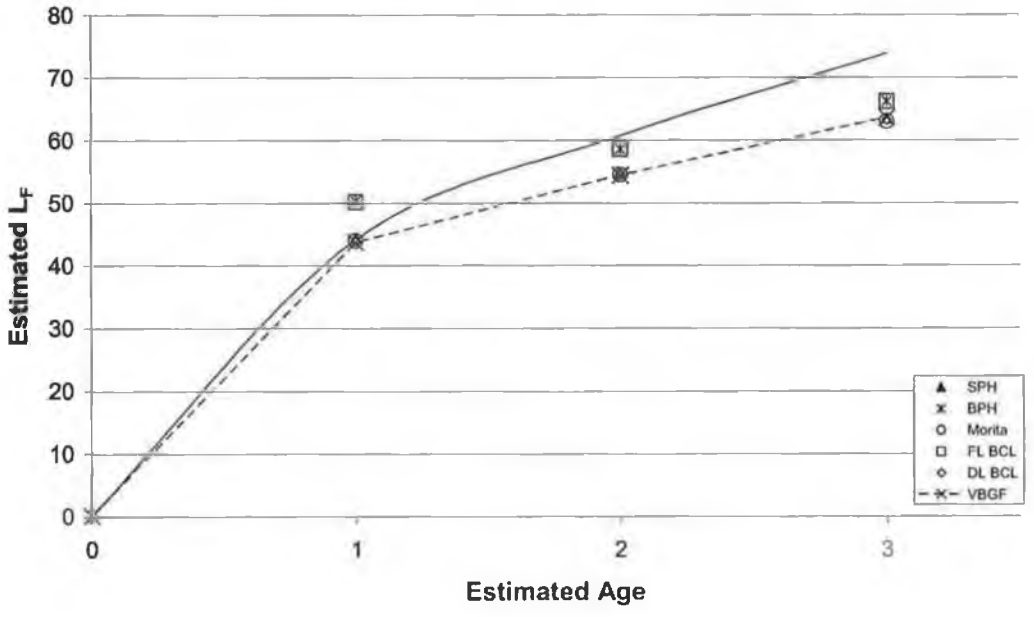

Figure 2.5. Estimated back calculated lengths from the SPH(▲), BPH (-), (Morita and Matsuishi, 2001) (○), Fraser-Lee Model (□) and the Dahl-Lea Model (◊) compared to estimated length at age based on ICCAT, 2003 using Von Bertalanffy (1938) (solid line) and the VBGC for samples in study (dashed line), intercept based on Piccinetti *et al*, 1973.

Chapter 3: Trace elements in the otoliths and dorsal spines of albacore tuna: an assessment of the effectiveness of cleaning procedures at removing post-mortem contamination.

3.1. Abstract

Trace element analysis or "elemental fingerprinting" is widely used in stock structure analyses (Thresher, 1999; Campana *et al.*, 2000). Post-mortem contamination of bony structures can confound the results of micro-constituent studies or introduce an additional source of noise in the data, thus reducing the ability of the technique to detect real variation in trace element concentrations. Despite the potential for post-mortem contamination during sample preparation, the effectiveness of the procedures used to remove potential contaminants from otoliths and other calcareous structure prior to laser ablation inductively coupled plasma mass spectrometry (LA ICPMS) has not previously been addressed. In this study, otoliths and dorsal spine sections of albacore tuna (*Thunnus alalunga*) collected from the North East Atlantic Ocean and the Mediterranean Sea were deliberately contaminated prior to analysis of trace element composition using LA ICPMS. The effectiveness three cleaning treatments (rinsing in ultra-pure water, 30% hydrogen peroxide and ultra-pure 5% nitric acid) at removing this post-mortem contamination were compared. Magnesium and strontium were relatively robust to post-mortem effects when exposed to contamination at concentrations of 50ppm and 200ppm respectively. Soaking in a solution containing ^{55}Mn, ^{118}Sn, ^{133}Cs and ^{137}Ba (50ppm) caused a marked increase in the detected concentration of each element in both structures. Translucent (slow growth) bands in both structures were more susceptible to contamination. Rinsing in ultra pure water or hydrogen peroxide was not effective at removing ^{55}Mn, ^{118}Sn, ^{133}Cs and ^{137}Ba contamination from either calcareous structure. Washing the otoliths and spines in nitric acid successfully removed post mortem contaminants.

The removal of otoliths from tuna damages the appearance of the fish and has an adverse effect on market value. Spines are easily removed, do not affect the appearance or value of the fish and are the most commonly used structure for age determination. In this study a weak but significant correlation was observed between ^{137}Ba in opaque zones in otoliths and dorsal spines. All other spine to otolith correlations was not significant. The results do not provide support for the use of spines as an alternative to otoliths in trace elemental analyses.

Keywords: Laser Ablation ICPMS, contamination, otoliths, spines, albacore

3.2. Introduction

The elemental composition of calcareous structures in fish (bones, otoliths, scales and fin spines) is affected by a variety of factors, both metabolic and abiotic, which are not completely understood (Bath *et al.*, 2000; Miller *et al.*, 2006; Lin *et al.*, 2007). As all the bony structures grow incrementally throughout the life of a fish (Campana and Neilson, 1985) they provide valuable information on the population dynamics of fish. By analysing the micro-elemental composition of calcareous structures (mainly otoliths) and relating this data to surrounding water chemistry (Farrell and Campana, 1996; Gillanders and Kingsford, 1996; Bath *et al.*, 2000; Milton and Chenery, 2001; Lin *et al.*, 2007), temperature (Kalish, 1989; Radtke *et al.*, 1990), and food sources (Gallahar and Kingsford, 1996), new information can be gathered on the life history of individuals or groups. Previous studies have described stock configurations (Campana *et al.*, 1994; Gillanders, 2001), site fidelity (Thorrold *et al.*, 1997), natal origin (Secor and Zdanowicz, 1998; Rooker *et al.*, 2001a; Brophy *et al.*, 2003; Rooker *et al.*, 2003) and migration patterns (Gemperline *et al.*, 2002; Stransky *et al.*, 2005) in different species of fish using this method.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is increasingly applied in investigations of fish stock structure. Material for ICPMS can be passed to the mass spectrometer after dissolution in an acid (solution based ICPMS) or by vaporisation with a laser (laser ablation ICPMS). Laser ablation ICPMS (LA ICPMS) has some merits over solution based; the entire sample is not destroyed by acid digestion and multiple data points can be obtained from one sample which can correspond to particular life history stages of the fish, e.g. the composition of the otolith primordium may reflect the environment of the recently hatched larva (Campana *et al.*, 1994). The samples can also be used in the cross calibration of different LA ICPMS machines and for age estimation. A comparative study by Ludsin *et al.* (2006) showed that solution based and laser ablation ICP MS both had merits and advantages when applied to different questions. Solution-based ICPMS is most commonly used for whole otolith analysis (Rooker *et al.*, 2001a; Rooker *et al.*, 2003; Swan *et al.*, 2006b), while LA ICP MS is the favoured technique for analyses that target particular points in the fish's life history and also for the analysis of very small larval otoliths (Brophy *et al.*, 2003; Ludsin *et al.*, 2006). Despite the

widespread application of the technique in studies of fish population structure and migration patterns, few studies have critically evaluated the protocols used for the collection, handling and processing of material for LA ICPMS, particularly in relation to the prevention or removal of contaminants.

Contamination of material collected for micro-chemical analysis can produce biased results and deviations from the natural composition. Elemental concentrations may be altered during different storage techniques of the whole fish or of the otoliths themselves. Brophy *et al.* (2003) demonstrated that prolonged freezing of the fish can result in elevated levels of Mg and Zn possibly through contamination from the endolymph. Milton and Chenery (1998) established that freezing, storing in alcohol and delaying the extraction of otoliths from fish dead for several hours had a small but significant effect on the concentration of some elements, especially Na and Mg. It is also possible that any tissue adhering to the structures when stored dry in paper envelopes can decompose and contaminate the samples. The collection of samples from commercial vessels is potentially problematic in terms of contamination of material (Milton and Chenery, 1998). There are usually no clean room facilities onboard for immediate extraction, increasing the possibility of contamination from endolymph fluid during storage. The range of different methods of storing fish onboard (on ice or frozen) may also lead to post-mortem variation in the composition of calcareous structures. It may be several hours or days from catching the fish to landing at port where otoliths or whole heads can be removed and stored. Extraction of otoliths may be undertaken in several laboratories with different working conditions. Additional difficulties are encountered when taking multiple measurements and samples from each fish (calcareous structures, muscle tissue, gonad tissue, and stomachs). In this case the time the otoliths remain within the fish can be variable and the possibility of contamination effects from the body fluids is increased (Milton and Chenery, 1998). These sources of error, although regrettable are not within the control of many researchers. During preparation for LA ICPMS structures are usually mounted in resin, sectioned to expose the internal zonation and polished to produce a smooth surface for ablation. Additional processing increases the possibility of contamination of the internal structure of dorsal spines and otoliths from the mounting medium, sectioning fluid, and contact with the diamond tipped copper sectioning blades (Arslan and Secor, 2008).

Several studies have investigated the effects of handling on whole otolith composition (Proctor *et al.*, 1995; Milton and Chenery, 1998; Swan *et al.*, 2006a) and compared the effectiveness of various cleaning methods at removing post mortem contamination prior to solution based ICPMS analysis (Rooker *et al.*, 2001a; Arslan and Secor, 2008). However very little has been published on the most appropriate way to deal with structures that will undergo LA ICPMS and the issue of contamination of polished sections has not been directly addressed (Arslan and Secor, 2008). As there are no definitive protocols, a variety of different cleaning methods have been applied to otoliths (whole and sectioned) analysed by LA ICPMS in previous studies. These include rinsing sections in deionised water (Stransky *et al.*, 2005); sonication and rinsing in deionised water (Thorrold *et al.*, 1997; Gemperline *et al.*, 2002); ultrasonically cleaning in 6% NaOCl followed by rinsing in ultra pure water (Ludsin *et al.*, 2006); rinsing in ultra pure water and pre-ablation by the first few shots of the laser then removed from subsequent analysis (Brophy *et al.*, 2003); and pre-ablation of the sample surface before analysis (Campana *et al.*, 1994). The effectiveness of these treatments at removing contaminants has not been assessed.

The first objective of this study is to examine the effects that three decontamination methods (rinsing with ultrapure water, 30% hydrogen peroxide and 5% nitric acid, as used in previous studies (Rooker *et al.*, 2001a; Stransky *et al.*, 2005)) have on calcareous structures which have been deliberately contaminated with a suite of chemicals (which have previously proved useful in microchemical analysis of stock composition (Rooker *et al.*, 2001b, Brophy *et al.*, 2003; Ludsin *et al.*, 2006)) at a level of 50ppm (^{24}Mg, ^{55}Mn, ^{118}Sn, ^{133}Cs and ^{137}Ba) and 200ppm (^{88}Sr) when analysed using a probe based analysis (LA ICPMS).

Otoliths are the preferred bony structure for micro-chemical studies due to their inert nature (Campana, 1999; Thresher, 1999). However, the removal of otoliths from high value species can diminish the market value of the fish. It is desirable therefore to ascertain whether comparable micro-elemental data can be obtained from using alternative structures to otoliths. Dorsal spine rays are easily removed and do not affect the value of the fish. They can be removed from living fish intended for release after tagging, or from individuals from rare or endangered stocks, where killing the fish for stock assessment is undesirable (Gillanders, 2001). The dorsal spine ray is composed of a softer calcium phosphate that can be reabsorbed by the animal (Gillanders, 2001). This impedes the reconstruction of early life histories

using micro-chemistry analysis of juvenile or larval cores. Previous studies have compared the microchemistry of otoliths with eye lenses (Dove and Kingsford, 1998), scales (Wells *et al.*, 2000) and dorsal spines (Gillanders, 2001) using solution based ICPMS and have demonstrated reasonably strong correlations in elemental composition across the three structures.

The second objective of this study is to assess the base composition of the dorsal spines of albacore tuna. Spines and otoliths from the same fish are compared to determine if their trace elemental concentrations are correlated with each other and to assess whether dorsal spines can be used as a proxy for otoliths in micro-elemental studies.

3.3. Materials and Methods

Sample Collection

Albacore were obtained onboard commercial vessels by Bord Iascaigh Mhara (BIM) in the Bay of Biscay and by Instituto Tecnológico Pesquero y Alimentario (ATZI), Spain from fish merchants in Southern Italy (Tyrrhenian Sea). Fish were caught by trolling in the North Atlantic Ocean and by pelagic long-lining in the Mediterranean Sea. Fifty fish were obtained from each area. Length, weight and maximum circumference measurements were taken (Table 3.1). The dorsal spines were removed from the fresh fish, scraped of flesh and placed in paper envelopes. The heads were severed and frozen for up to 3 months before removal of the otoliths in the laboratory. Once extracted, the macula tissue on the otolith was removed using forceps and the otoliths were soaked in ultra pure water to remove any adhering tissue or fluids. They were then dried before storing in sterile acid washed plastic tubes for transport.

Sample Preparation

A sub-sample of sixteen fish (eight from each area) was used in this experiment. The first dorsal spine and one randomly selected otolith from each fish were imbedded in Buehler Epo-thin® low viscosity epoxy resin. Three 1mm and five 0.75mm transverse sections were made of each spine and otolith, respectively, on a Buehler Isomet® Low Speed Saw using Buehler Iso Cut Fluid as a blade lubricant, according to protocols in Doray *et al.* (2004) and Oritz de Zárate *et al.* (2007a). Sections were washed in ultra pure water to remove any adhering sectioning fluid, then dried and stored in acid cleaned plastic tubes. The otolith sections were subsequently polished on 600, 2500 and 4000 grit silicon carbide wet-dry polishing paper using 3µm diamond paste and ultra pure water with a final polish on Buehler micro cloth.

The analysis was carried out in two stages. The purpose of the first experiment (Trial 1) was to assess the effect of contamination on the elemental concentrations in each structure and to determine the effectiveness of removing contaminants by cleaning with ultra-pure water. Samples were deliberately contaminated by soaking in a solution spiked with salts of ^{24}Mg, ^{55}Mn, ^{88}Sr, ^{133}Cs and

^{137}Ba at a concentration of 50ppm for each element. Caesium was added to the mixture as it is naturally found in very low concentrations in the structures and is therefore an ideal marker to ensure that contamination solution was absorbed by the structure. In the second part (Trial 2) of the investigation three different cleaning methods were compared for their success at returning elemental concentrations to the baseline levels detected in the control (uncontaminated) samples. In trial 2 ^{118}Sn was added to the contamination solution as an additional potential contaminant, and the concentration of ^{88}Sr was increased in the contamination salt solution to 200ppm.

Rooker *et al.* (2001b) suggested that work on the handling and cleaning protocols on otoliths should utilise pairs of otoliths for comparisons. This study used multiple sections from a single otolith and dorsal spine, and thus removed the effect of individual variation in the base composition of otoliths or spines (Campana *et al.*, 1994). As multiple sections of one structure was used in this experiment the number of individuals necessary was reduced to sixteen individuals in total. Each otolith and spine section was subjected to one of five treatments to allow paired comparisons of treatment effects (Table 3.2). Each control section was prepared by washing in ultra pure water and drying in a laminar flow hood. The remaining four sections from each otolith and spine were immersed for 2 minutes in the contamination solution; those in treatment 2 were dried in the laminar flow hood and the remaining 3 sets were allowed to air dry overnight before cleaning. Sections in treatment 3 were subsequently triple rinsed in ultra pure water, while those in treatment 4 were immersed in 30% hydrogen peroxide for 2 minutes then flooded with three washes of ultra pure water. Sections subjected to treatment 5 were cleaned in 5% analytical grade nitric acid for 2 minutes and flooded with three washes of ultra pure water to remove any acid residue. Sections in all treatments were placed in a laminar flow hood to dry for 8 hours. Once dry the sections were stored in acid washed plastic tubes until the analysis. It was established by weighing that approximately 5% of material of both structures was lost through the decontamination process using 5% nitric acid. There was no other discernable loss of sample material using either of the other cleaning methods.

Elemental Analysis - Calcium Quantification

A randomly selected sub-sample of five dorsal spines and five otoliths from each area was prepared for inductively coupled plasma atomic emission spectrometry (ICP-AES) to ascertain percentage values for the total calcium. The otolith and spine samples were weighed then transferred to glass test tubes. 1ml of Aristar nitric acid was added and the tube gently heated to 40°C. Once the samples had dissolved fully they were cooled and made up to 25ml with deionised water. Samples were analysed on a Varian Vista-Pro ICP-AES, which had been calibrated with synthetic standards. A pure calcium carbonate sample was dissolved in the same way and analysed to check the accuracy of this method. An average value of 35% and 16% total calcium was present in otoliths and dorsal spines, respectively. The percentage of CaO (the preferred expression of calcium concentration, Jeffries, pers. comm.) was calculated from these values (49% and 22.4%, respectively) and subsequently utilized in the data processing as an internal standard to compensate for the poor precision in the analytical signal caused by variations in the mass of material ablated (Campana *et al.*, 1994). A constant concentration of CaO was assumed across the entire matrix of both structures.

Elemental Analysis - Laser Ablation

Optimum conditions for laser ablation were ascertained by ablating sites of the spine and otolith sections that did not interfere with the subsequent analysis; a balance was achieved between high levels of $^{43}Ca/^{88}Sr/^{24}Mg$ and low levels of the other elements. Optimum detection limits were achieved using a laser spot size of 40µm, laser firing rate of 10Hz and a fluence of 0.3 J/cm^2. Samples were analysed using a New Wave Research (California) frequency quintupled Nd:YAG laser (λ = 213nm) coupled with a VG Elemental Plasma Quad 3 Inductively Coupled Plasma Mass Spectrophotometer (Thermo Elemental, Cheshire). Ablated material was carried via an argon helium air flow to the ICPMS which was alight throughout the day to avoid any contaminant build up of the Ni cones (Campana, 1999). Samples under investigation were mounted on a slide (47mm x 25mm) in a Perspex ablation cell, which was placed on a motorized stage under a transmitted light microscope. Real time images were viewed on a computer screen via a CCD camera connected to the microscope. The ablation

sites were marked out prior to analysis within the laser settings program. The standard reference material (SRM) used was glass reference NIST 612 from the National Institute of Standards and Technology (Gaithersberg, Maryland, USA). Samples were analysed in sets of 20, the SRM was analysed twice at the start of a run and twice at the end to account for any instrumental drift. The duration of the data acquisition was 90 s. Background counts of each isotope in the gas blank (Ar/He mixture) were run in the initial 40 s prior to ablation and subtracted from that ablation. Data were collected in peak jumping mode as a function of time (time-resolved analysis). Samples were analysed in a random order to avoid any potentially confounding effects of variation in operating conditions between runs. Theoretical limits of detection (LOD) for each element were calculated from the mean of the blank signal plus three standard deviations. LOD's were normalized within each run for the ablation yield to account for the variation in sensitivity associated with the differences in amount of material ablated.

Two sites were ablated on each sample; 150μm and 300μm from the edge in the mid plane of the spines and 80μm and 160μm from the edge of the ventral arm of the otolith section (Figure 3.1). These points were targeted as they corresponded to the most recent opaque and translucent bands in each structure, and corresponding points in the life history of the fish as deposited in the otoliths and spines. The locations of ablation sites relative to opaque and translucent increments were confirmed after the analysis through examination under a stereoscopic microscope. Data were processed using laser ablation specific macros within the package, Lotus 1-2-3. The minor isotope of calcium, ^{43}Ca, was used as the internal standard as determined from the ICP-AES. During data collection the contamination spike could be seen in real-time on a graphical display on the ICPMS control computer. During data processing the first 5-10 seconds of ablation was excluded in an attempt to remove surface contamination and average count rates for each element recorded during the subsequent 15 to 20 s of stable signature. Percentage relative standard deviations (% R.S.D.) based on replicate measurements of the calibration standards reflect the level of precision achieved for each element. Mean % R.S.D. and L.O.D. are shown in Table 3.3.

Statistical Analysis

Data were classified on the basis of whether the point of ablation was on an opaque or translucent incremental band. Points of ablation that spanned both bands were excluded from analysis. Data for opaque and translucent increments were analysed separately to determine if the uptake of contaminants and the effectiveness of cleaning procedures were influenced by structural differences in the calcium carbonate matrix between these areas (Gauldie and Nelson, 1988; Gauldie and Radtke, 1990). All data were examined for outliers by subtracting the mean and dividing by the standard deviation and comparing these results to Grubb's critical values (URL 7). Any data points with values greater than the Grubb's critical values were standardized (Windsorized) against the next highest or lowest value in that data set. The number of standardized outliers and percentages of samples with concentrations above the limits of detection for each element are shown in Table 3.3. When the recorded elemental concentration was below the limit of detection that reading was discarded and was not included in subsequent data analysis. Equal variances across groups and normality within groups were assessed for each set of pairwise differences between treatments using Levene's statistic and Anderson-Darling test, respectively. A significance level of $\alpha = 0.05$ was used in all tests. Data were transformed, using the inverse or $\log_{10}$ functions as appropriate, to conform to homogeneity of variance and normality. Paired t-tests were used to compare treatment and control (treatment 1) sections. Data that did not show equal variances or normality after transformation were analysed using the non-parametric Wilcoxon's signed rank test (Minitab® version 15.0). Coefficient of variance was calculated for each element and treatment to assess the variability in counts per second across ablation transects. As the concentration of ^{133}Cs is very low or negligible, it was often below the detection limits in treatment 1. Therefore ^{133}Cs was treated as a categorical variable with two levels (above LOD and below LOD). Chi Square analysis was used to determine if ^{133}Cs concentrations above LOD were more frequent in structures from treatment 2 than in structures from treatment 1, and thus indicate if the structures had absorbed the contamination solution.

Correlations between Otolith and Dorsal Spine Ray Microchemistry

The control samples (treatment 1) were also used to assess the correlations between elemental concentrations in otoliths and dorsal spine rays. Data obtained from opaque (fast) and translucent (slow) growth areas were analysed separately. Matching pairs of data (within an individual fish) for each element were checked for correlation using Pearson's correlation statistic in Minitab® v. 15.

3.4. Results

Trial 1 - Contamination in Otoliths

Mean concentrations of elements in otoliths from treatments 1, 2 and 3 (Trial 1) are shown in Table 3.4. The results of the statistical comparisons of elemental concentrations in otoliths from treatments 1-3 are shown in Table 3.6. The mean concentration of ^{133}Cs in otoliths from treatment 1 was 0.02ppm and increased to 0.21ppm in otoliths from treatment 2. Chi square comparisons of ^{133}Cs concentrations (frequencies above LOD and below LOD) between treatment 1 and 2 showed significant differences ($P<0.05$); the number of data points above detection limits were much higher in treatment 2 compared to treatment 1. Paired t-tests comparing the composition of otoliths from treatment 1 and treatment 2 confirmed that the concentrations of ^{55}Mn in opaque bands and ^{133}Ba in both opaque and translucent bands ($P<0.05$, Table 3.6) were increased by the contamination treatment. A non-parametric Wilcoxon's signed rank test found no significant difference in ^{55}Mn concentration between treatments 1 and 2 in translucent bands in otolith, however only two comparisons were available for this element at this location, as six of the data points in treatment 1 were below detection limits. Chi square analysis indicates significant differences in the number of detections above the LOD in ^{55}Mn in translucent bands in treatment 2 compared to treatment 1 ($P<0.05$), suggesting that the contamination treatment did increase manganese concentrations in the translucent bands. Concentrations of ^{88}Sr and ^{24}Mg in otoliths did not change after soaking in the spiked solution indicating that otoliths are robust to post-mortem contamination when exposed to these elements at a concentration of 50ppm ($P>0.05$, Table 3.6).

Paired comparisons of otoliths from treatments 1 and 3 indicated that after cleaning with ultra pure water, the concentration of ^{55}Mn was significantly higher than control levels in translucent regions, while ^{137}Ba remained elevated in both opaque and translucent regions ($P<0.05$, Table 3.6). This indicates that cleaning with ultra-pure water is not effective at removing contamination caused by these elements from otoliths. Ablation profiles are shown for an uncontaminated otolith (Figure 3.2a) and a contaminated then cleaned with ultrapure water (treatment 3) otolith (Figure 3.2b). A clear increase or spike of elements is evident in Figure 3.2b between 40 and 50s, indicating the presence of contamination on the structure surface. Although this spike

was removed from the signal during data processing, elemental concentrations remained elevated.

Trial 1 - Contamination in Dorsal Spines

Average elemental concentrations within dorsal spine rays from treatments 1, 2 and 3 (Trial 1) are shown in Table 3.5. The results of the statistical comparisons of elemental concentrations in spines from treatments 1-3 are shown in Table 3.7. The mean concentration of ^{133}Cs was 0.13ppm in spines from treatment 1 and 1.20ppm in spines from treatment 2. Chi square analysis revealed that there were significantly more data points above the detection limits for spines from treatment 2 compared to treatment 1 ($P<0.05$). Pairwise comparisons showed that contamination (treatment 2) increased the concentrations of ^{55}Mn and ^{137}Ba in the both opaque and translucent bands ($P<0.05$, Table 3.7). Concentrations of ^{24}Mg in translucent bands was significantly lower in spines from treatment 2 compared to treatment 1, suggesting that soaking in the contamination solution reduced their concentration ($P<0.05$, Table 3.7). There was no evidence of contamination with ^{24}Mg in opaque regions or ^{88}Sr in either opaque or translucent regions, again indicating resilience to this level of contamination for these elements ($P>0.05$, Table 3.7). Pairwise comparisons indicated that for ^{24}Mg there were significant differences between treatments 1 and 3, however mean concentrations were lower in treatment 3 compared to treatment 1 (Figure 3.4). The comparison of treatments 1 and 3 showed that rinsing in ultrapure water was not sufficient to return ^{55}Mn and ^{137}Ba concentrations in either opaque or translucent regions to base levels ($P<0.05$, Table 3.7).

Trial 2 – A Comparison of Cleaning Methods in Otoliths

In the ablation profiles (e.g. Figure 3.2b) a contamination spike appears at ~40s (when the firing of the laser on the sample is initiated) which is reduced by 50s. This spike was consistently removed during data processing; any differences between treatments are deeper within the otolith matrix rather than purely surface contamination.

Pairwise comparisons showed that the concentration of ^{24}Mg in translucent bands was significantly higher for otoliths from treatment 3 compared to otoliths from treatment 1 ($P<0.05$, Table 3.8). This indicates that unlike in trial 1, magnesium

concentrations in the otolith were altered by the contamination treatment and that ^{24}Mg contamination at 50ppm was not removed from this portion of the otolith by washing with ultrapure water. No significant differences in ^{24}Mg concentrations were observed between treatments 4 and treatment 1 or between treatments 5 and 1 in either opaque or translucent increments ($P>0.05$, Table 3.8), showing that any ^{24}Mg contamination absorbed by the otolith was removed by hydrogen peroxide and nitric acid baths.

The concentration of ^{55}Mn in both opaque and translucent bands was significantly higher in otoliths from treatments 3 and 4 compared to otoliths from treatment 1 ($P<0.05$, Table 3.8). This suggests that rinsing in ultrapure water or hydrogen peroxide is not effective at removing ^{55}Mn contamination (Figure 3.3). Paired t-tests found no significant difference in ^{55}Mn concentrations between otoliths from treatment 5 and treatment 1 ($P>0.05$, Table 3.8) indicating that cleaning with nitric is effective at removing ^{55}Mn contamination in both opaque and translucent bands. The mean ^{55}Mn concentration in otoliths from treatment 5 is much reduced compared to the mean ^{55}Mn concentration in otoliths from treatments 3 and 4, and is much closer to the mean concentration observed in otoliths from treatment 1 (Figure 3.3).

In trial 1, the contamination treatment did not increase the concentration of ^{88}Sr in the otolith. This may have been caused by the low concentration of strontium in the contamination solution (50ppm) relative to the base concentration in the otolith (1200-2400ppm). In trial 2, the strontium concentration in the contamination solution was increased to 200ppm. Pairwise comparisons revealed no significant differences in ^{88}Sr concentrations in otoliths from treatment 1 compared to otoliths from treatments 3, 4 or 5, for both opaque and translucent bands ($P>0.05$, Table 3.8). This suggests that if the otolith did absorb any strontium from the more concentrated contamination solution, this was effectively removed by all of the three cleaning treatments.

Concentrations of ^{118}Sn (inversely transformed) were significantly higher in otoliths from treatment 3 and 4 compared to otoliths from treatment 1 ($P<0.05$ Table 3.8), indicating that cleaning with ultrapure water or hydrogen peroxide was not effective at removing contamination for this element. A reduction in ^{118}Sn was observed after cleaning in nitric acid (treatment 5, Figure 3.3) however, ^{118}Sn concentrations in opaque increments were still significantly different from control

concentrations in treatment 1 ($P<0.05$, Table 3.8). No significant differences were observed in translucent increments in treatment 5 versus treatment 1 ($P>0.05$, Table 3.8).

^{133}Cs was only present above detection limits in contaminated samples. The removal of this element was not compared across cleaning methods as it is not detectable in most otoliths, and is not of analytical interest in micro-elemental studies. However it may be worth noting that once present ^{133}Cs persists at greater concentrations compared to baseline concentrations (Figure 3.3), however nitric acid did remove most of the ^{133}Cs contamination.

Pairwise comparisons showed that ^{137}Ba concentrations in contaminated otoliths remained significantly higher than concentrations in the control otoliths (treatment 1) after cleaning with ultra pure water (treatment 3) ($P<0.05$, Table 3.8). ^{137}Ba concentrations remained elevated after cleaning with hydrogen peroxide (treatment 4, Figure 3.3). This difference was statistically significant for opaque bands ($P<0.05$, Table 3.7) and just below significance for translucent bands ($P=0.06$, Table 3.8). Concentrations of ^{137}Ba in otoliths cleaned with nitric acid (treatment 5) were not significantly different to the concentrations in the control otoliths for either opaque or translucent bands ($P>0.05$, Table 3.8). This indicates that cleaning with nitric acid is the most effective cleaning method for removing barium contamination.

Trial 2 – A Comparison of Cleaning Methods in Dorsal Spines

Paired t-tests revealed no significant difference in ^{24}Mg concentrations in spines from treatment 1 compared to treatments 3, 4 or 5 ($P>0.05$, Table 3.9). However, the concentration of ^{24}Mg in the contamination solution was low (50ppm) compared to naturally occurring levels in the spine ($2707 \mu g\ g^{-1} \pm 542\ \mu g\ g^{-1}$). The impact of this contaminant on the spine would be low and may not be detectable. It is therefore not possible to accurately assess the effectiveness of the three cleaning methods at removing magnesium contamination from dorsal spines. However, the concentrations of ^{24}Mg in treatment 3 are reduced compared to treatment 1; this suggests that this element may not be firmly bound to the matrix in spines and is therefore easily removed.

The non-parametric Wilcoxon's signed rank tests showed that ^{55}Mn concentrations in spines remained elevated above control levels (treatment 1) after

cleaning with ultra pure water (treatment 3) and hydrogen peroxide (treatment 4) ($P<0.05$, Table 3.9). Nitric acid appeared to effectively remove ^{55}Mn contamination from the spines; a paired t-test showed no significant difference in the concentration of ^{55}Mn in spines from treatments 5 compared to spines from treatment 1 ($P>0.05$, Table 3.9, Figure 3.4).

^{88}Sr concentrations in treated spines were not significantly different from concentrations in the control spines after cleaning with ultra pure water and hydrogen peroxide for either opaque or translucent bands ($P>0.05$, Table 3.9). However, compared to control spines, concentrations were significantly lower in translucent bands after cleaning with the nitric acid wash ($P<0.05$, Table 3.9, Figure 3.4), suggesting that nitric acid is causing an unnecessary removal of ^{88}Sr from the spine material.

Paired comparisons indicated significant differences in ^{118}Sn concentrations between spines from treatment 1 and spines from treatments 3, 4 and 5 for both opaque and translucent bands ($P<0.05$, Table 3.9). However mean concentrations (Figure 3.4) of ^{118}Sn were considerably closer to control concentrations in spines from treatment 5 compared to spines from treatments 3 and 4, indicating that nitric acid partly removed this contamination and a longer soaking time may further reduce this or remove this contamination.

As with the otoliths ^{133}Cs was not considered of interest in the decontamination procedure as natural concentrations of this element are very low. However ^{133}Cs was not removed by any of the treatments (Figure 3.4).

The non-parametric Wilcoxon's signed rank test indicated significant differences between the concentrations of ^{137}Ba in spines from treatment 1 compared to spines from treatments 3 and 4 for both opaque and translucent bands ($P<0.05$, Table 3.9). This difference is also shown in Figure 3.4 where levels of ^{137}Ba remain elevated after washing in water or hydrogen peroxide. Paired t-tests revealed no significant differences in ^{137}Ba concentrations between spines from treatment 1 and spines from treatment 5 for both opaque and translucent bands ($P>0.05$, Table 3.9). This indicates that rinsing in nitric acid is effective at removing barium contamination.

Correlation between Otolith and Dorsal Spine Ray Microchemistry

Differences were observed in the total percentage of calcium by ICP-AES analysis of the otoliths and dorsal spines with 49% and 22.4% each, respectively. A higher mean concentration of ^{24}Mg was observed in the dorsal spines at 2708 ± 542ppm compared to an average of 22 ± 8ppm in otoliths. Manganese was also found in greater concentrations in the dorsal spines at a mean of 5.63 ± 1.72ppm compared to 0.59 ± 0.25ppm observed in otoliths. In contrast to this ^{88}Sr was found in higher concentrations in the otoliths (1823 ± 347ppm) versus dorsal spines (548 ± 74ppm). ^{118}Sn concentrations were also greater in otoliths compared to dorsal spines at 2.83 ± 1.45ppm and 1.73 ± 0.25ppm, respectively. The concentrations of ^{137}Ba were similar in both structures at 1.03 ±0.40ppm and 1.11 ± 0.38ppm.

A weak but significant correlation was observed between the ^{137}Ba in opaque growth bands between otoliths and dorsal spines (Table 3.10). All other elemental and growth band correlations were not significant ($P<0.05$, Table 3.10). The correlations were weak with Pearson's correlation coefficients ranging from <0.01 to 0.33, however the highest Pearson's correlation coefficients were observed in opaque growth increments (Table 3.10).

3.5. Discussion

Caesium occurs at naturally low levels in otoliths and spines and was below the limits of detection in most of the untreated structures. The occurrence of detectable levels of this elemental marker in the treated spines confirmed that the contamination solution had been absorbed by the structures and that their base composition had been altered. Soaking in the contamination solution also increased the concentrations of manganese, tin and barium in the otoliths and spines, showing that for these elements, exposure of otoliths and dorsal spine sections to contamination at a concentration of 50ppm can change the concentrations detected by LA ICPMS analysis. The contamination treatment had little impact on the concentrations of magnesium or strontium in otoliths or spines, suggesting that exposure to contamination at concentrations of 50ppm (magnesium) and 200ppm (strontium) does not affect the detections of these elements in LA ICPMS analysis.

Proctor and Thresher (1998) suggested that group II divalent ions, which substitute easily into the aragonite matrix, and are relatively reliable for stock delineation. Miller *et al.* (2006) subsequently observed that manganese, a divalent ion, has a weak chemical binding and is not associated with the protein matrix of cod (*Gadus morhua*) otoliths. If an element has a weak binding then it may be subject to greater rates of transfer or substitution (Burton, 1994; Thresher, 1999), and hence contamination from external sources. Certain elements may be more susceptible to this and differences in the composition of calcareous structures may make them predisposed to different contamination effects by the same contaminant (Dove *et al.*, 1996). This study has shown that manganese and barium, both divalent ions, are susceptible to contamination affects when exposure to contaminants in spite of predictions of robustness by Proctor and Thresher (1998).

The base composition of otoliths is difficult to discern (Proctor and Thresher, 1998) and true contaminating effects complicated to detect. In this study, exposure to contaminants at concentrations 10 – 100 times greater than the concentrations naturally present in the structures increased the amount of manganese and barium detected by a factor of 1.5 – 5 (see Table 3.5). Rooker *et al.* (2001b) showed that exposure to contamination at a level of 10ppm altered the concentrations of manganese and barium detected using solution based ICP-MS; in that case the

relationship between the contamination solution and the natural levels of the elements in the otolith were approximately 12:1 (in the case of manganese) and 8:1 (in the case of barium). Any natural sources of contamination which expose otoliths and spines to these concentrations could produce a contamination affect. Potential sources of contamination for otoliths and spines include exposure to water, blood or endolymph or cross-contamination from metal implements (Kalish, 1989; 1991; Gauldie *et al.*, 1998; Thresher, 1999). Manganese concentrations in seawater is at least an order of magnitude lower than base concentrations in otoliths or spines, ranging from 0.08ppb to 10ppb (Turekian, 1976; Bruland *et al.*, 1991) therefore post-mortem exposure to seawater is unlikely to affect the detection of manganese. Barium has been shown to have a mean concentration of 0.02ppm in seawater (Turekian, 1976), so this element is also unlikely to affected by post-mortem contamination with seawater. The exact concentration of manganese and barium in the endolymph is difficult to measure because of the small volume enclosed in the otic cleft (i.e. the cavity in the skull enclosing the otolith), and the difficulties associated with obtaining measurements from the endolymph shortly after death (Romanek and Gauldie, 1996). A study by Melancon *et al.* (2009) shows that the concentrations of magnesium and barium in blood and endolymph are at least an order of magnitude higher than the concentrations in seawater. In the two species of freshwater fish examined, the concentration of manganese in the endolymph is 0.3 to 1 times that in the otolith while barium endolymph concentrations are 0.02-0.2 times that in the otolith (Melancon *et al.*, 2009). Endolymph concentrations of these elements are therefore still much lower relative to otolith concentrations compared to the contamination solutions used in this and previous studies. Nonetheless, exposure to body fluids is a more likely source of post-mortem contamination than exposure to seawater.

The concentration of strontium in seawater has been shown to range between 8.0 and 8.2ppm (Carr, 1970; Turekian, 1976), this is similar to the peak level of strontium (7.4ppm) found in endolymph fluid of bearded rock cod by Kalish (1991). Strontium concentrations were found at greater concentrations in blood plasma ranging between 8ppm and 18ppm (Kalish, 1991). However, none of these fluids show a high enough concentration to suggest potential post-mortem contamination of strontium in either otoliths or spines. It is probable that otolith and spines are unlikely to be exposed to levels of strontium large enough to cause any contamination effect through natural sources.

Although the contamination treatment in this study had little impact on magnesium concentrations, there is some evidence that magnesium in translucent bands in both structures was affected by post-mortem contamination. This may be due to differences in the calcium carbonate matrix of opaque and translucent bands (Gauldie and Nelson, 1988; Gauldie and Radtke, 1990). Rooker *et al.* (2001b) also observed increased magnesium concentrations in otoliths exposed to contamination at a concentration of 10ppm. In spines, cleaning in ultrapure water appeared to reduce magnesium concentrations relative to concentrations in control sections. Interestingly Rooker *et al.* (2001b) observed a similar effect of cleaning on magnesium concentrations, where concentrations in cleaned otoliths were less than observed in control otoliths, although drew no conclusions from this observation. Magnesium is present in seawater at a concentration of 1,290ppm (Turekian, 1976), therefore post-mortem exposure to seawater is a potential source of contamination. Magnesium is also known to be present in the endolymphatic fluid. Concentrations of 0.16ppm are reported for rattails (*Coryphaenoides rupestris*), 1.06ppm for rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*), (Romanek and Gauldie, 1996) 2.84ppm for burbot (*Lota lota*) and 4.89 for lake trout (*Salvelinus namaycush*) (Melancon *et al.*, 2009). Although the contamination risk is not as high as for seawater, endolymphatic fluid may also contain magnesium at high enough concentrations to pose a threat of contamination. Both Milton and Chenery (1998) and Brophy *et al.* (2003) have shown that post-mortem handling especially freezing can alter the concentration of Mg in otoliths possibly from the endolymphatic tissue; therefore this source of contamination should not be disregarded.

The simplest decontamination method applied in this study was rinsing the samples in ultrapure water. Previous studies by Thorrold *et al.* (1997), Gemperline *et al.* (2002) and Stransky *et al.* (2005) have employed this cleaning method on otoliths analysed using LA ICPMS. Our results indicate that this cleaning method is not suitable at removing contamination in either calcareous structure.

Rinsing with hydrogen peroxide was not effective at removing contamination in this study. Concentrations of manganese, tin and barium remained at similar concentrations to samples cleaned with ultra-pure water in both otoliths and dorsal spines. It is therefore concluded that hydrogen peroxide is not suitable as a cleaning agent for removing potential contamination from fish calcareous structures, although

this solution is used for removing adhering tissue from the calcareous structures (Rooker *et al.*, 2001b).

Rinsing in nitric acid was the most effective cleaning method for removing contaminants from otoliths and spines. After acid washing, concentrations of manganese and barium decreased to concentrations that were indistinguishable from the concentrations in untreated otoliths and spines. The removal of ^{118}Sn was ineffective in both structures using all three cleaning treatments; however this element is rarely of interest in stock discrimination studies as it is considered to be a ubiquitous contaminant (Edmonds *et al.*, 1992; Gillanders and Kingsford, 1996; Rooker *et al.*, 2001a). The persistence of tin contamination in the otoliths and spines after acid washing confirms that its inclusion in trace elemental analysis of fish hard parts is undesirable.

Pre-ablation of samples, by firing the laser at the sample surface prior to sample collection is used in some studies as a decontamination method, where samples are too small or delicate to subject to other decontamination methods (Campana *et al.*, 1994; Brophy *et al.*, 2003). In this study, the first 5 to 10 seconds of the signal were removed during data processing in an attempt to remove the contamination spike from all structures. The persistence of contaminants in structures cleaned with water or hydrogen peroxide, even after this data processing step, indicates that pre-ablation is not effective at removing contaminants within the otolith matrix.

In a study on specimen handling and otolith preparation, Proctor and Thresher (1998) concluded that otoliths (and to that effect spines) are porous and are sensitive to the effects of post-mortem handling procedures. Gauldie *et al.* (1998) have shown that fluid can very easily move through the otolith crystalline structure. Nitric acid is an aggressive caustic agent, and although it has been shown to be effective at removing contaminants, prolonged immersion or excessively high concentration may cause disintegration of the calcium carbonate matrix of the calcareous structures. Although Rooker *et al.* (2001b) showed that contamination of magnesium, manganese, and barium at a level of 10ppm was effectively removed from otoliths by a combination of soaking in 3% hydrogen peroxide followed by immersing in 1% nitric acid they also indicated minor but consistent losses in concentrations of magnesium and manganese in their decontamination procedure. In this study approximately 5% of material (by weight) was lost from spines and otoliths after

cleaning in nitric acid. This highlights the problem of compromise between maximising the removal of contaminants while minimizing the degradation of the sample.

Small inconsistencies were observed in treatments between the opaque and translucent growth bands within the otoliths and dorsal spines. Specifically, there was some evidence that the contamination treatment altered magnesium concentrations in translucent bands of otoliths and spines, while no change was observed in opaque bands. The inconsistencies observed in different treatments between opaque and translucent growth increments may reflect the differences in the protein – crystalline carbonate matrices deposited during different phases of the fish's growth (Campana and Neilson, 1985; Gauldie and Nelson, 1988; Oritz de Zárate *et al.*, 2007a). Differences in the deposition rate and crystal habits have been observed in opaque and translucent bands of otoliths in other species (Gauldie, 1991), the carbonate crystals deposited may behave differently when exposed to contaminating influences (Burton, 1994).

The differences in elemental concentration between the hard metabolically inert calcium aragonite of the otoliths and the softer more permeable calcium phosphate of the spines may occur due to differences in the metabolic pathways within the fish, which are not completely understood (Campana, 1999). Strontium was found at a lower concentration in dorsal spines compared to otoliths, which may be linked to the lower concentration of Ca present in spines (16% compared to 35% average in albacore otoliths), as strontium can substitute for calcium in calcium carbonate matrices (Campana, 1999). The higher percentage of calcium in otoliths compared to dorsal spines may be due to the difference in structural matrix and re-absorption of the dorsal spine in albacore (Gauldie *et al.*, 1992; Megalofonou, 2000). The poor correlations observed in this study between otolith and spine elemental signatures, may be due to the ablation points not corresponding to the exact same time in the fish's life history. The temporal stability of elemental concentrations in soft tissues is also questionable (Campana and Gagne, 1995). Given the absence of a strong correlation between otolith and spine concentrations, any stock differences observed in otolith elemental concentrations would not be detected in spines, at least for the elements analysed here. Until the process of elemental incorporation into spines is more fully understood and the stability of these elements in the structure

confirmed, dorsal spines are not recommended as an alternative to otoliths in trace elemental studies for albacore tuna.

In conclusion sample preparation and the use of decontamination solutions can have an effect on the microchemistry of calcareous structures. Strontium is robust to contamination at 200ppm and the natural sources of contamination reviewed here do not exceed 20ppm. Magnesium is resistant to contamination at 50ppm; however natural sources and post-mortem storage can cause adverse changes in base concentrations. Both otoliths and spines are susceptible to contamination of manganese and barium at a concentration of 50ppm; however these occur naturally at much lower levels. Decontamination using ultrapure water, hydrogen peroxide and pre-ablation are not suitable for removing contaminating elements from otoliths and dorsal spines. Nitric acid was effective at removing the majority of contaminants, with the exception of tin indicating that this method can be used to prepare archived samples but care is necessary not to compromise the structural integrity of the sample by overuse of aggressive cleaning methods. This study has shown that these certain elements can be confidently used in future LA ICPMS studies of otoliths and dorsal spine rays, as their relatively high concentrations in the structures make them less susceptible to post-mortem alteration from natural sources. The comparisons of the cleaning agents indicate that different elements are effectively removed by different cleaning agents, therefore future decontamination procedures would be dictated by the elements of interest.

Table 3.1. Mean fork length, weight and circumference of total and sub samples of fish from the North East Atlantic Ocean and Mediterranean Sea.

Origin	*N*	Mean ± S.E L_F (mm)	Mean ± S.E. Weight (kg)	Mean ± S.E. Circumference (mm)
Total				
NE Atlantic	57	595 ± 11	5.28 ± 0.34	389 ± 8
Mediterranean	50	645 ± 6	5.06 ± 0.14	422 ± 4
Sub sample				
NE Atlantic	8	663 ± 39	7.47 ± 1.11	446 ± 25
Mediterranean	8	650 ± 13	5.25 ± 0.29	426 ± 8

Table 3.2. List of treatments applied to sections of sagittal otolith and dorsal spine rays.

Treatment	
1	Control: rinsed in ultra pure water and dried after sectioning
2	Contaminated by soaking in contamination solution
3	Contaminated, dried and washed with ultra pure water
4	Contaminated, dried and cleaned using 30% hydrogen peroxide solution
5	Contaminated, dried and cleaned using 5% nitric acid

Table 3.3. Relative Standard deviations (%R.S.D.), limits of detection (LOD), % of readings > LOD, and number of Windsorized outliers for all elements recorded during two separate analyses (in Trial 1 and 2) for albacore otolith and dorsal spine rays. Note: ^{133}Cs was only present in contaminated samples; it does not occur naturally in otoliths and spines.

	^{24}Mg	^{55}Mn	^{88}Sr	^{118}Sn	^{133}Cs	^{137}Ba
Trial 1						
%RSD	1.72	1.93	1.19	1.88	5.86	1.20
LOD (ppm)	0.41	0.12	0.02	0.16	0.01	0.06
%>LOD	86	86	100	100	84	100
Outliers	0 (0%)	6 (4.7%)	0 (0%)	8 (6.3%)	0 (0%)	7 (5.5%)
Trial 2						
%RSD	2.31	0.85	1.32	1.24	2.79	1.65
LOD (ppm)	1.62	0.07	0.01	0.11	0.00	0.03
%>LOD	100	98	100	100	84	100
Outliers	0 (0%)	4 (2.5%)	0 (0%)	4 (2.5%)	1 (0.6%)	3 (1.9%)

Table 3.4. Average concentration of elements of interest in translucent and opaque bands in otoliths for treatments 1-3 (Trial 1) and treatments 1, 4-6 (Trial 2). Concentrations are given in ppm (μg g^{-1}) ablated material ± 1 SD.

		^{24}Mg	^{55}Mn	^{88}Sr	^{118}Sn	^{133}Cs	^{138}Ba
Trial 1							
Treatment 1	Opaque Bands	19.72± 4.58	0.54± 0.20	1764± 362	1.89± 0.37	0.02± 0.01	0.86± 0.30
	Translucent Bands	20.69± 3.93	0.48± 0.06	1696± 327	1.96± 0.31	-	0.84± 0.17
Treatment 2	Opaque Bands	21.06± 3.82	1.12± 0.54	1957± 443	2.51± 0.78	0.21± 0.19	1.52± 0.69
	Translucent Bands	21.71± 3.62	1.00± 0.36	1877± 330	2.48± 0.76	0.25± 0.13	1.54± 0.69
Treatment 3	Opaque Bands	19.33± 1.98	0.87± 0.21	1813± 314	2.03± 0.20	0.13± 0.07	1.74± 0.35
	Translucent Bands	19.79± 3.04	1.01± 0.44	1636± 298	2.10± 0.15	0.10± 0.07	1.49± 0.36
Trial 2							
Treatment 1	Opaque Bands	17.67± 7.40	0.49± 0.33	1833± 347	1.28± 0.20	0.07± 0.12	1.29± 0.51
	Translucent Bands	19.16± 6.99	0.38± 0.21	1868± 277	1.51± 0.46	0.07± 0.14	1.39± 0.53
Treatment 3	Opaque Bands	20.14± 4.63	4.11± 3.36	1825± 276	5.67± 3.11	0.69± 0.85	3.30± 2.18
	Translucent Bands	22.69± 8.50	3.82± 3.07	2155± 414	6.16± 4.27	0.79± 1.11	3.11± 1.65
Treatment 4	Opaque Bands	21.84± 4.39	4.96± 3.76	1880± 343	5.61± 3.43	0.31± 0.43	3.16± 2.09
	Translucent Bands	22.91± 5.03	4.76± 3.70	1873± 326	6.55± 3.18	0.37± 0.48	3.37± 2.29
Treatment 5	Opaque Bands	19.70± 2.53	1.53± 0.17	1919± 256	2.61± 2.17	0.17± 0.15	0.48± 0.17
	Translucent Bands	20.43± 4.55	0.46± 0.10	2014± 257	1.58± 0.32	0.23± 0.19	1.36± 0.37

Table 3.5. Average concentration of elements of interest in translucent and opaque bands in dorsal spine rays for treatments 1-3 (Trial 1) and treatments 1, 4-6 (Trial 2). Concentrations are given in ppm ($\mu g\ g^{-1}$) ablated material ± 1 SD.

		^{24}Mg	^{55}Mn	^{88}Sr	^{118}Sn	^{133}Cs	^{138}Ba
Trial 1							
Treatment 1	Opaque Bands	2702± 687	5.36± 1.85	563± 21	1.66± 0.16	0.01± <0.01	0.95± 0.23
	Translucent Bands	2768± 374	5.72± 1.60	589± 78	1.65± 0.21	0.01± <0.01	1.13± 0.31
Treatment 2	Opaque Bands	2661± 788	8.57± 2.10	549± 100	1.90± 0.23	0.37± 0.37	4.39 2.22
	Translucent Bands	2206± 399	10.02± 3.57	648± 152	1.85± 0.24	0.61± 0.61	3.50± 1.79
Treatment 3	Opaque Bands	2755± 606	8.18± 2.31	584± 106	1.76± 0.36	1.09± 0.98	3.60± 2.23
	Translucent Bands	2336± 329	7.09± 2.59	606± 136	1.72± 0.08	1.61± 2.43	2.03± 0.74
Trial 2							
Treatment 1	Opaque Bands	2706± 519	6.2± 1.9	480± 37	1.82± 0.28	-	1.04± 0.27
	Translucent Bands	2599± 611	5.3± 1.6	489± 65	2.28± 0.91	-	1.20± 0.25
Treatment 3	Opaque Bands	2540± 348	105± 82	582± 82	31.27± 24.43	1.52± 0.88	41.41± 35.14
	Translucent Bands	2448± 221	102± 86	605± 143	30.27± 25.73	1.81± 0.53	42.17± 36.43
Treatment 4	Opaque Bands	2649± 386	140± 110	570± 92	32.25± 15.38	1.89± 0.88	42.15± 23.49
	Translucent Bands	2538± 296	120± 98	540± 73	35.00± 19.68	1.54± 0.52	40.46± 22.61
Treatment 5	Opaque Bands	2990± 725	8.2± 4.2	488± 70	3.16± 1.45	1.79± 0.85	1.48± 0.38
	Translucent Bands	2817± 215	6.8± 3.5	480± 51	2.82± 0.90	1.70± 0.68	1.45± 0.67

Table 3.6. Pair wise comparisons (paired t-test) indicating differences for each element between treatments (2 vs.1 and 3 vs.1) in Trial 1, divided into opaque and translucent bands in otoliths.

		^{24}Mg	^{55}Mn	^{88}Sr	^{137}Ba
		Treatment 2 vs. Treatment 1			
Opaque Bands	Test Statistic	0.72	2.81	0.47	2.46
	P Value	0.50	0.03	0.66	0.04
	Paired Comparisons	7	7	7	7
Translucent Bands	Test Statistic	1.45	**3.0**	1.31	3.8
	P Value	0.22	**0.37**	0.26	0.02
	Paired Comparisons	5	**2**	5	5
		Treatment 3 vs. Treatment 1			
Opaque Bands	Test Statistic	-0.16	2.21	0.57	8.03
	P Value	0.88	0.09	0.59	<0.01
	Paired Comparisons	6	5	6	6
Translucent Bands	Test Statistic	-0.81	3.54	2.24	5.83
	P Value	0.45	0.02	0.07	<0.01
	Paired Comparisons	7	5	7	7

Table 3.7. Pair wise comparisons (paired t-test and Wilcoxon's signed rank test (non-parametric results shown in bold)) indicating differences for each element between treatments (2 vs.1 and 3 vs.1) in Trial 1 and divided into opaque and translucent bands in dorsal spine rays.

		^{24}Mg	^{55}Mn	^{88}Sr	^{137}Ba
		Treatment 2 vs. Treatment 1			
Opaque Bands	Test Statistic	0.06	2.95	-0.32	**21**
	P Value	0.96	0.03	0.76	**0.04**
	Paired Comparisons	6	6	6	**6**
Translucent Bands	Test Statistic	-4.53	2.47	0.54	3.67
	P Value	<0.01	0.04	0.61	0.01
	Paired Comparisons	7	7	7	7
		Treatment 3 vs. Treatment 1			
Opaque Bands	Test Statistic	-0.87	2.34	0.47	4.26
	P Value	0.42	0.07	0.66	<0.01
	Paired Comparisons	6	6	6	6
Translucent Bands	Test Statistic	-2.33	3.33	0.35	3.64
	P Value	0.06	0.02	0.74	0.01
	Paired Comparisons	7	7	7	7

Table 3.8. Pair wise comparisons (paired t-test) indicating differences for each element for cleaning treatments versus control in Trial 2, divided into opaque and translucent bands in otoliths.

		^{24}Mg	^{55}Mn	^{88}Sr	^{118}Sn	^{137}Ba
		Treatment 3 vs. Treatment 1				
Opaque Bands	Test Statistic	1.84	36	0.88	16.48	6.7
	P Value	0.11	0.01	0.41	<0.01	<0.01
	Paired Comparisons	8	8	8	8	8
Translucent Bands	Test Statistic	2.5	28	1.84	5.81	8.91
	P Value	0.04	0.02	0.11	<0.01	<0.01
	Paired Comparisons	8	8	8	8	8
		Treatment 4 vs. Treatment 1				
Opaque Bands	Test Statistic	0.56	35	0.05	11.82	2.54
	P Value	0.59	0.02	0.96	<0.01	0.04
	Paired Comparisons	8	8	8	8	8
Translucent Bands	Test Statistic	0.85	36	0.62	7.39	2.23
	P Value	0.42	0.01	0.56	<0.01	0.06
	Paired Comparisons	8	8	8	8	8
		Treatment 5 vs. Treatment 1				
Opaque Bands	Test Statistic	1.65	16	0.91	2.74	2.12
	P Value	0.14	0.30	0.39	0.03	0.07
	Paired Comparisons	8	8	8	8	8
Translucent Bands	Test Statistic	0.76	25	1.17	0.94	0.62
	P Value	0.47	0.08	0.28	0.38	0.55
	Paired Comparisons	8	8	8	8	8

Table 3.9. Pair wise comparisons (paired t-test and Wilcoxon's signed rank test (non-parametric results shown in bold)) indicating differences for each element for cleaning treatments versus control in Trial 2, divided into opaque and translucent bands in dorsal spine rays.

		^{24}Mg	^{55}Mn	^{88}Sr	^{118}Sn	^{137}Ba
		Treatment 3 vs. Treatment 1				
Opaque Bands	Test Statistic	-0.84	**36**	0.79	**35**	**36**
	P Value	0.43	**0.01**	0.46	**0.02**	**0.01**
	Paired Comparisons	8	8	8	8	8
Translucent Bands	Test Statistic	-2.66	**34**	-0.1	**36**	**36**
	P Value	0.03	**0.03**	0.93	**0.01**	**0.01**
	Paired Comparisons	8	8	8	8	8
		Treatment 4 vs. Treatment 1				
Opaque Bands	Test Statistic	-0.84	**36**	1.35	**36**	**36**
	P Value	0.43	**0.01**	0.22	**0.01**	**0.01**
	Paired Comparisons	8	8	8	8	8
Translucent Bands	Test Statistic	-0.18	**36**	0.28	**36**	**36**
	P Value	0.86	**0.01**	0.79	**0.01**	**0.01**
	Paired Comparisons	8	8	8	8	8
		Treatment 5 vs. Treatment 1				
Opaque Bands	Test Statistic	0.43	1.19	-2.1	4.27	1.6
	P Value	0.69	0.28	0.08	0.01	0.16
	Paired Comparisons	7	7	7	7	7
Translucent Bands	Test Statistic	0.72	0.66	-4.1	4.07	0.4
	P Value	0.50	0.53	0.01	0.01	0.7
	Paired Comparisons	7	7	7	7	7

Table 3.10. Pearson's correlations and corresponding significance of otolith vs. dorsal spine ray comparisons for opaque and translucent areas.

		^{24}Mg	^{55}Mn	^{88}Sr	^{118}Sn	^{137}Ba
Opaque Bands	Pearson's Correlation	0.33	<0.01	0.21	0.25	0.50
	P Value	ns	ns	ns	ns	0.01
Translucent Bands	Pearson's Correlation	0.13	-0.19	0.19	-0.22	0.29
	P Value	ns	ns	ns	ns	ns

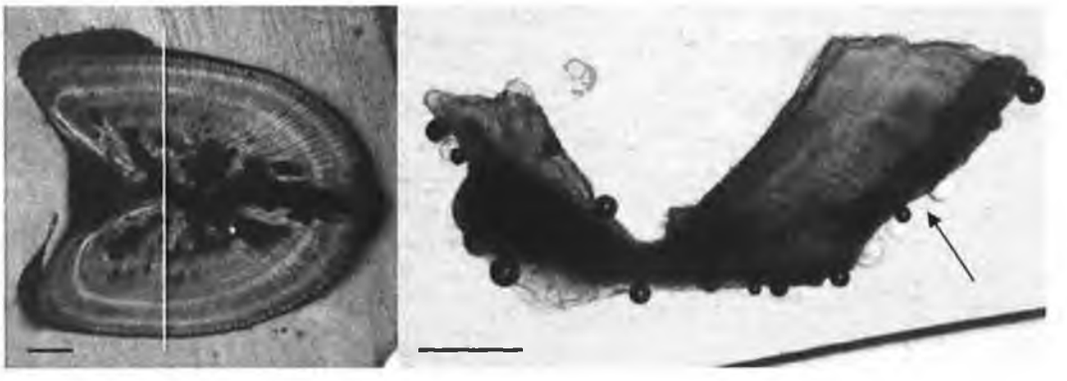

Figure 3.1. Stereoscopic view of transverse section of first dorsal spine ray (left at x1.6 Mag., the white line indicates the mid-plane of the spine) and sagittal otolith (right at x4 Mag., arrow indicates the ventral arm of the otolith). Scale bars shown at bottom left, 500µm in both images.

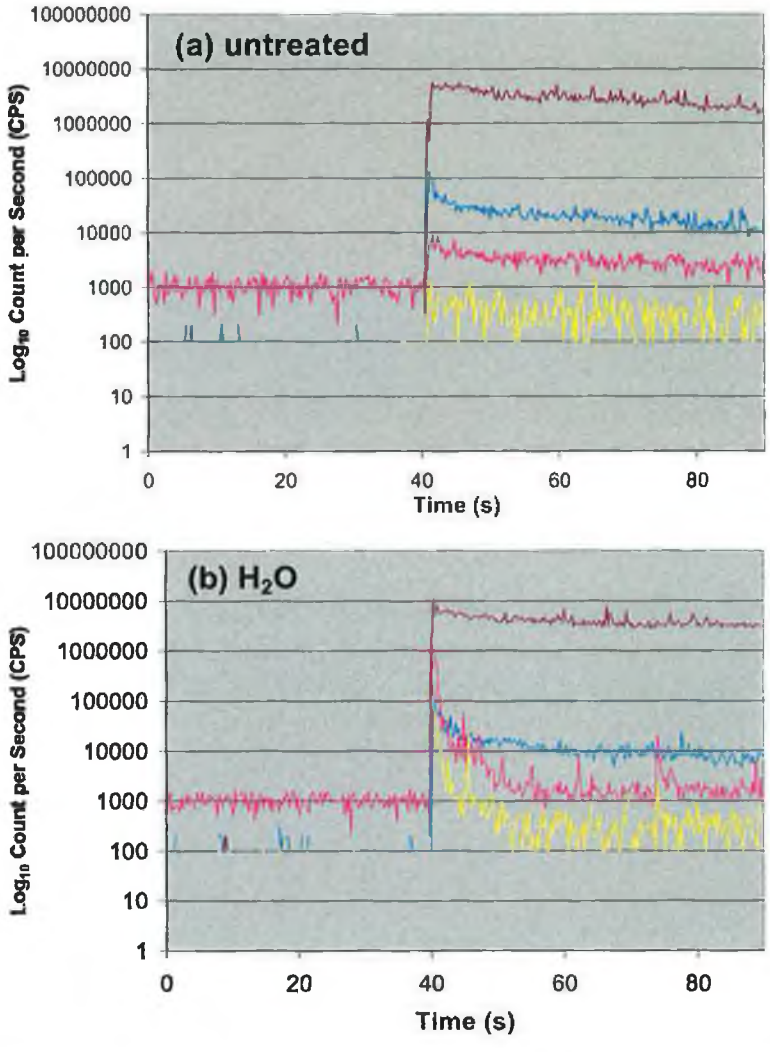

Figure 3.2. Ablation profiles showing the concentrations of ^{24}Mg (blue), ^{55}Mn (pink), ^{88}Sr (purple), and ^{137}Ba (yellow) on a) an untreated otolith, b) a contaminated otolith cleaned with H_2O.

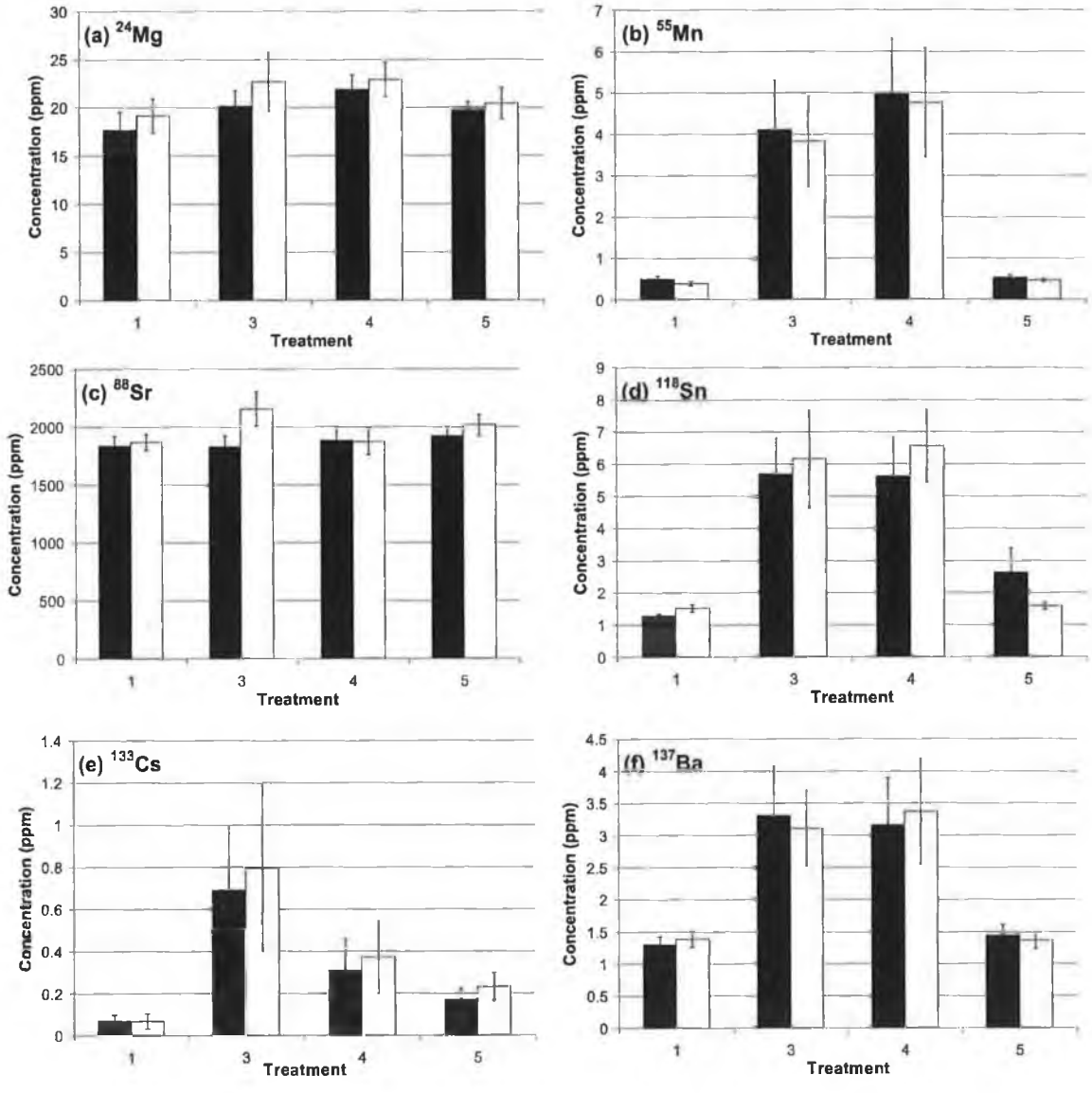

Figure 3.3. Mean concentrations of six elements ± SE in opaque bands (shaded bars) and translucent bands (clear bars) of albacore otoliths treated in different contamination/cleaning methods. (a) ^{24}Mg, (b)^{55}Mn, (c) ^{88}Sr, (d) ^{118}Sn, (e) ^{133}Cs and (f) ^{137}Ba.

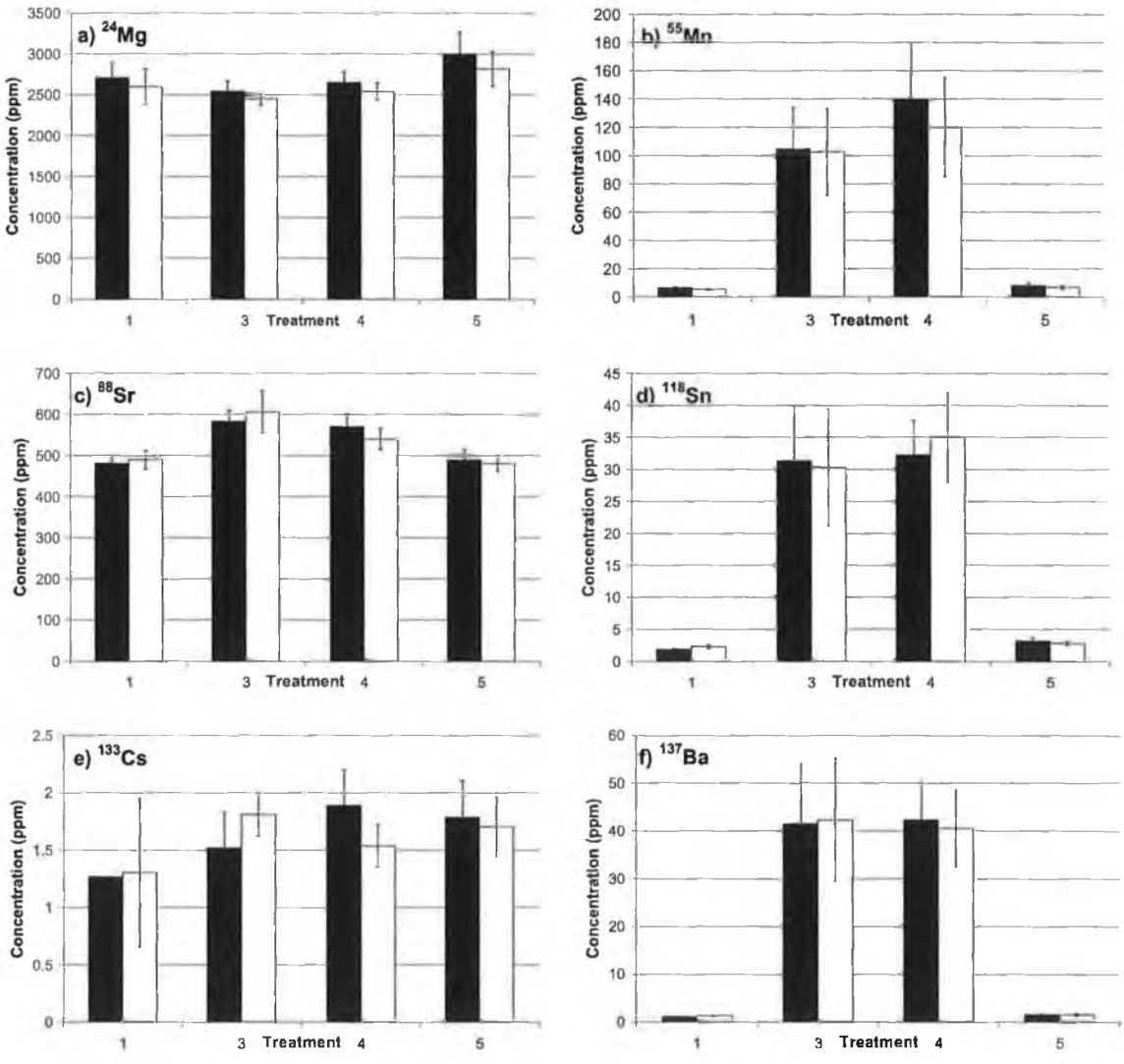

Figure 3.4. Mean concentrations of six elements ± SE in opaque bands (shaded bars) and translucent bands (clear bars) of albacore dorsal spine rays treated in different contamination/cleaning methods. (a) ^{24}Mg, (b)^{55}Mn, (c) ^{88}Sr, (d) ^{118}Sn, (e) ^{133}Cs and (f) ^{137}Ba.

Chapter 4: Cross-species amplification and characterization of thirty-three microsatellites in albacore tuna, *Thunnus alalunga*.

4.1. Abstract

Cross-species amplification of microsatellite loci is widely applied in fish population studies, where microsatellites have not been developed for the study species. Thirty-three microsatellite loci isolated from bluefin tunas, *Thunnus thynnus thynnus* and *T. thynnus orientalis* were tested for cross-species amplification with albacore tuna, *Thunnus alalunga*. Amplification success was 100% with mean observed heterozygosity of 0.693 and numbers of alleles per locus ranging from 2 to 24, averaging 12.27 alleles. Fifteen loci across two study areas showed departures from Hardy-Weinberg equilibrium. This information should prove useful in choosing microsatellite loci in studies of population genetic structure of albacore.

Keywords: *Thunnus alalunga*, albacore, tuna, cross-amplification, microsatellite

4.2. Short Communication

Tunas of the genus *Thunnus* have a cosmopolitan distribution throughout the world's oceans in both the Northern and Southern hemispheres. There are six species and two sub species in the genus. One of these, albacore tuna (*Thunnus alalunga*, Bonnaterre, 1788), is found in the sub-tropical and temperate zones of all oceans. This species accounts for approximately 5% of global catches (at 0.2 million tonnes) of the major tunas; and approximately 15% of tuna catches in the Atlantic Ocean (Miyake *et al.*, 2004). The spawning stock biomass of Atlantic albacore has declined in recent years (ICCAT, 2007; Anon, 2009). Albacore is classed as "data deficient" on the IUCN Red list (Uozumi, 1996); in the Atlantic the north stock is classified as vulnerable and the south stock is critically endangered. To date, there is little information on the genetic structure of the species. Therefore, information on population genetic structure using a suite of polymorphic microsatellite markers should prove valuable for proper management and conservation of the species.

Currently, 36 microsatellite loci have been developed for bluefin tuna *Thunnus thynnus thynnus* and *T. thynnus orientalis* (Takagi *et al.*, 1999; McDowell *et al.*, 2002; Clark *et al.*, 2004) but none have been developed specifically for albacore. No precise information has been provided on the usefulness and variability of the 36 loci in other tuna species, with the exception of Takagi *et al.* (2001) who conducted a preliminary genetic study on inter-oceanic albacore populations using four microsatellite loci (*Ttho*1, *Ttho*4, *Ttho*6 and *Ttho*7). Due to the presence of null alleles at two (*Tth*10-43 and *Tth*23-6) of the 36 bluefin microsatellite loci (Clark *et al.*, 2004) these loci were not screened in our study; an additional locus (*Tth*114) did not amplify in albacore.

Two Northern hemisphere populations of albacore were sampled during 2006 from the Bay of Biscay (N=50) in the North Atlantic, and the Tyrrhenian Sea (N=50) in the Mediterranean. All individuals were screened for the 33 loci. The International Commission for the Conservation of Atlantic Tunas (ICCAT) considers these to be two separate populations on the basis of geographic isolation and the rarity of migrations through the straits of Gibraltar (ICCAT, 1996).

Genomic DNA was extracted from muscle tissue using the phenol-chloroform method of Sambrook *et al.* (1989). PCR was carried out using a reaction volume of 10µl, containing 0.17U *Taq* polymerase, 1 X reaction buffer (Bioline), 0.25 µM of

each primer (the reverse primer was labelled with either IRD 700 or 800 dye), 2µl of mixed dNTPs, 0.2 mM $MgCl_2$ and 1µl of template DNA. Thermocycling procedures followed those in Takagi *et al.* (1999), McDowell *et al.* (2002) and Clark *et al.* (2004), depending on the locus. Amplification products diluted with bromo-phenol blue in formamide loading buffer were separated on 6% polyacrylamide gels using a Li-Cor 4300 automated sequencer (Li-Cor, Lincoln, NE, USA) with a size standard (50-350 base pairs, Li-Cor, Lincoln, NE, USA) run in the centre and at both ends of the gels to calibrate allele size. An internal marker consisting of individuals where allele sizes had been pre-determined was included to ensure consistency in genotype scoring across runs. Fragment length polymorphisms were scored with GENE IMAGIR software (Li-Cor, Lincoln, NE, USA).

All 33 screened loci were polymorphic in the two populations, with numbers of alleles ranging from two to 23, and observed heterozygosity from 0.320 to 0.958 with a mean value of 0.693 (Table 4.1). Heterozygosities and allele numbers were similar to those reported for bluefin tunas (Takagi *et al.*, 1999; McDowell *et al.*, 2002; Clark *et al.*, 2004). There were, however, some notable exceptions: *Tth*17 where a tenfold higher number of alleles was detected in albacore compared to bluefin, and *Ttho*4, *Tth*5, *Tth*39, *Tth*185, *Tth*226 and *Tth*265 where the number of alleles was two or more times higher. At locus *Ttho*6 the number of alleles was half that reported by Takagi *et al.* (2001), although *Ttho*1 and *Ttho*7 show comparable allele numbers, ranges and heterozygosities for the same study area. Tests for conformance to Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium between pairs of loci, were performed using GENEPOP v. 3.4 (Raymond and Rousset, 1995) with specified Markov chain parameters (10000 dememorization steps, 100 batches, 5000 iterations per batch). Significant deviations from Hardy-Weinberg equilibrium were observed for the same six loci in the two populations, and for an additional five loci in the Bay of Biscay population and for four loci in the Mediterranean population (Table 4.1). Only one of 496 locus pairs, between *Tth*178 and *Tth*265 in the Mediterranean sample, showed significant linkage disequilibrium, after Bonferroni correction (Rice, 1989). The program MICRO-CHECKER (Van Oosterhout *et al.*, 2004) indicated that deficits were likely attributable to null alleles at 12 of the 33 loci. Null alleles were not detected for any of these loci in bluefin tuna (McDowell *et al.*, 2002; Clark *et al.*, 2004) based on F_{IS} estimations.

This study highlights the benefit of testing for cross-species amplification of microsatellite loci that have been specifically developed for one or two species, but not tested in other closely related species. There is now a set of microsatellite loci available that can be utilised in albacore genetic population studies to help resolve stock heterogeneity across the species distribution.

Table 4.1. Summary data for cross amplification of loci in albacore tuna (*Thunnus alalunga*, Bonn. 1788).

Locus	Primer Sequence(5'-3')	Species of Isolation (Source)	Population	N/N_A	Size Range (bp)	Heterozygosity: H_E/H_O	P_{HW}	Evidence of Null Alleles
Ttho1	F: AAACGCTCCAGGCAAATGAC	*T. thynnus orientalis*	Atlantic	50/7	157-185	0.588/0.560	**0.046**	No
	R: CATAGCACACCCATAGACAC	(Takagi *et al*, 1999)	Mediterranean	50/8	169-185	0.439/0.380	**0.040**	No
Ttho4	F: CCTTCATCTTCAGTCCCATC	*T. thynnus orientalis*	Atlantic	50/21	138-200	0.903/0.840	0.055	No
	R: CTGTTCATCTGTTCGCCC	(Takagi *et al*, 1999)	Mediterranean	50/21	138-196	0.897/0.780	**0.000**	Yes
Ttho6	F: TTCTGCTTCTTTCTTCTGG	*T. thynnus orientalis*	Atlantic	50/11	133-157	0.742/0.660	0.634	No
	R: GAAAACACAGGGATTATGG	(Takagi *et al*, 1999)	Mediterranean	50/9	135-157	0.746/0.720	0.204	No
Ttho7	F: ACTGGATGAAAGGCGATTAC	*T. thynnus orientalis*	Atlantic	50/13	178-210	0.855/0.840	0.531	No
	R: ACAGAGGAGCATAACAGAAAC	(Takagi *et al*, 1999)	Mediterranean	50/9	188-210	0.785/0.820	0.891	No
Tth4	F: GAAACGCAGCCGGAGAGGAAAGAG	*Thunnus thynnus thynnus*	Atlantic	48/24	196-248	0.939/0.958	0.071	No
	R: AATGTGAGGGGGATGGGAGCTTGT	(Clark *et al*, 2004)	Mediterranean	49/22	196-244	0.928/0.918	0.910	No
Tth5	F: AGGGGGTGGACAAAATAAAAGG	*T. thynnus thynnus*	Atlantic	49/19	121-213	0.898/0.918	0.768	No
	R: TGGGAGTGGAGAATGACAGGAGAG	(McDowell *et al*, 2002)	Mediterranean	49/17	121-213	0.901/0.837	**0.040**	No
Tth8	F: CCTGTTTGAGTGTTTATCTGTGCG	*T. thynnus thynnus*	Atlantic	50/8	302-330	0.831/0.820	0.281	No
	R: GGTGTTGGCTATTGAGGAAATGC	(McDowell *et al*, 2002)	Mediterranean	50/9	298-330	0.834/0.800	0.843	No
Tth10	F: GCTGAGCACGCATTTACTGTAG	*T. thynnus thynnus*	Atlantic	50/2	120-124	0.440/0.440	1.000	No
	R: CGTCACAACCTTCCAACTCG	(McDowell *et al*, 2002)	Mediterranean	50/2	120-124	0.358/0.380	1.000	No

*Tth*14	F: AAGATGGGGGTACAAACAAG	*T. thynnus thynnus*	Atlantic	50/6	126-138	0.694/0.540	0.065	Yes
	R: TTTTTCATACCGGAGGACTC	(Clark *et al*, 2004)	Mediterranean	50/5	126-138	0.603/0.600	0.699	No
*Tth*17	F: TCTGTGAGCATCACGTTACTG	*T. thynnus thynnus*	Atlantic	50/22	93-147	0.943/0.880	**0.029**	No
	R:TCTGAAGCGACTGCATTCT	(Clark *et al*, 2004)	Mediterranean	50/18	97-147	0.916/0.860	0.324	No
*Tth*21	F: GACAGAGAGACAGAGAGAAGGGGAG	*T. thynnus thynnus*	Atlantic	50/4	117-129	0.533/0.480	0.747	No
	R: CACAGAGTTGATAACAGCGGCAG	(McDowell *et al*, 2002)	Mediterranean	50/3	117-129	0.575/0.520	0.668	No
*Tth*34	F: GATGCCATTTCTCTGTCTATCTG	*T. thynnus thynnus*	Atlantic	50/11	87-131	0.715/0.600	0.136	No
	R: AAGCCGTTCCCTCAGTGTC	(McDowell *et al*, 2002)	Mediterranean	50/6	91-131	0.649/0.680	0.829	No
*Tth*38	F: ACAAGCAGCCATAGAGCAGCAC	*T. thynnus thynnus*	Atlantic	50/6	167-187	0.692/0.640	**0.021**	No
	R: CAACAAGCAAAATGACCGCC	(McDowell *et al*, 2002)	Mediterranean	50/7	175-207	0.712/0.680	0.771	No
*Tth*39	F: GAGAACGAACAGACGGACCAAC	*T. thynnus thynnus*	Atlantic	50/2	106-110	0.379/0.420	0.705	No
	R: GCAATCCCACTCAGACTTCCTTC	(McDowell *et al*, 2002)	Mediterranean	50/2	106-110	0.311/0.340	0.668	No
*Tth*62	F: GGTATATGTGTTTGTTAGGCGTGTG	*T. thynnus thynnus*	Atlantic	50/8	85-103	0.658/0.400	**0.000**	Yes
	R: TTTTCCCAATGCGACTGATGA	(Clark *et al*, 2004)	Mediterranean	50/8	83-101	0.724/0.380	**0.000**	Yes
*Tth*112	F: TAGCAACAAGCAGTTAGAGA	*T. thynnus thynnus*	Atlantic	50/20	113-173	0.921/0.760	**0.000**	Yes
	R: GAAAGTCCTATCAATCAAG	(Clark *et al*, 2004)	Mediterranean	50/20	113-157	0.914/0.800	**0.029**	Yes
*Tth*152	F: ATGCCGCTCTGATGAGGTTA	*T. thynnus thynnus*	Atlantic	50/5	167-177	0.346/0.360	0.637	No
	R: CCTGTTCTTCCCCGACACTG	(Clark *et al*, 2004)	Mediterranean	50/4	171-177	0.458/0.320	**0.020**	Yes

*Tth*157	F: CAAGAGGCTTAAAGCAAACTC	*T. thynnus thynnus*	Atlantic	50/6	117-131	0.511/0.580	0.900	No
	R: CATGAATGGGTTCCTTCATC	(Clark *et al*, 2004)	Mediterranean	50/6	117-129	0.509/0.520	0.145	No
*Tth*178	F: AGACATCTGCAGGAAGTG	*T. thynnus thynnus*	Atlantic	50/11	120-154	0.834/0.740	0.236	No
	R: AAAGAACTGCAACATGACA	(Clark *et al*, 2004)	Mediterranean	50/8	140-154	0.781/0.700	0.871	No
*Tth*185	F: AGCCTTCGATGCACCCGTCTTAC	*T. thynnus thynnus*	Atlantic	50/23	152-210	0.926/0.940	0.764	No
	R: ATCATGCTTCCACTGCCCACTCTC	(Clark *et al*, 2004)	Mediterranean	50/17	142-210	0.901/0.920	0.395	No
*Tth*204	F: CCTGTGGAGCCATGACAG	*T. thynnus thynnus*	Atlantic	50/13	147-169	0.851/0.740	**0.000**	Yes
	R:TTTAATTTCCCTAGTGTCCTGAT	(Clark *et al*, 2004)	Mediterranean	50/12	143-169	0.818/0.700	0.345	Yes
*Tth*207	F: GTGTGTCGGATGCTGATT	*T. thynnus thynnus*	Atlantic	50/3	272-276	0.519/0.520	1.000	No
	R: ATAGATGGATTGTTATGTCTTGT	(Clark *et al*, 2004)	Mediterranean	50/3	272-276	0.516/0.640	0.114	No
*Tth*208	F: GAGAGGGAAAGCAAAGAAG	*T. thynnus thynnus*	Atlantic	50/21	150-190	0.910/0.700	**0.017**	Yes
	R: GTTGAGCTGCTGACACAGA	(Clark *et al*, 2004)	Mediterranean	50/19	152-190	0.928/0.820	**0.000**	Yes
*Tth*211	F: ATAAACACACCCTTACTCACT	*T. thynnus thynnus*	Atlantic	50/12	181-209	0.825/0.760	0.579	No
	R: TATTCTTTTCCTAACCATTCT	(Clark *et al*, 2004)	Mediterranean	50/9	181-209	0.852/0.760	0.332	No
*Tth*217	F: ACTTTCCCTACCTGGTGATAT	*T. thynnus thynnus*	Atlantic	50/23	215-271	0.949/0.840	0.076	Yes
	R: GTTGTAACCACTAATGGTAAAC	(Clark *et al*, 2004)	Mediterranean	50/23	215-273	0.938/0.780	**0.005**	Yes
*Tth*226	F: ATTGTGCATACACCAACAC	*T. thynnus thynnus*	Atlantic	50/20	141-187	0.942/0.860	**0.009**	No
	R:ACACTGTGCTGAACTCAACTTA	(Clark *et al*, 2004)	Mediterranean	50/18	141-187	0.915/0.860	0.146	No

*Tth*254	F: TGGGAGACAGTGACATACGAG	*T. thynnus thynnus*	Atlantic	50/20	81-151	0.915/0.840	0.079	No
	R: CACACCAAACAAAGGATTACT	(Clark *et al*, 2004)	Mediterranean	50/19	81-151	0.897/0.780	**0.023**	Yes
*Tth*260	F: TTTATCCTCAGATTTGATATG	*T. thynnus thynnus*	Atlantic	50/18	90-156	0.908/0.880	0.689	No
	R: GTGTCTGCTTGTATTTGTGT	(Clark *et al*, 2004)	Mediterranean	48/18	86-144	0.904/0.958	0.068	No
*Tth*265	F: TCGGTGGGAGGGAGACGC	*T. thynnus thynnus*	Atlantic	50/23	185-245	0.943/0.800	**0.000**	Yes
	R: CAGGTGGTGCAATTAATGGAAAA	(Clark *et al*, 2004)	Mediterranean	50/16	185-245	0.917/0.840	0.459	No
*Tth*1-31	F: ATGCACAAGTCATTTATCACCT	*T. thynnus thynnus*	Atlantic	50/12	94-122	0.843/0.860	0.794	No
	R: AGATGCATGGATTACATTCTACC	(Clark *et al*, 2004)	Mediterranean	50/18	92-124	0.812/0.740	0.139	No
*Tth*7-16	F: TTCCTTCAGGACCAATAAAGTATC	*T. thynnus thynnus*	Atlantic	48/14	94-162	0.867/0.583	**0.000**	Yes
	R: TCAGAGCTGCTAGCATGTATGTAG	(Clark *et al*, 2004)	Mediterranean	49/14	94-150	0.857/0.551	**0.001**	Yes
*Tth*12-29	F:CATACATACACATCTACATTGAACG	*T. thynnus thynnus*	Atlantic	50/8	93-111	0.652/0.760	0.942	No
	R: CACCAACAAGTACTGTAGATATGC	(Clark *et al*, 2004)	Mediterranean	50/10	91-113	0.703/0.720	0.144	No
*Tth*16-2	F: TGAGTTCCCAATTACACT	*T. thynnus thynnus*	Atlantic	50/6	91-103	0.612/0.620	**0.008**	No
	R: CTGTAGCATCGTCACAGT	(Clark *et al*, 2004)	Mediterranean	50/8	89-103	0.619/0.500	**0.002**	Yes

N, number of individuals assayed; N_A, number of alleles detected; H_E and H_O, expected and observed heterozygosity respectively; P_{HW}, probability that genotype proportions conform to expectations of Hardy-Weinberg equilibrium (P<0.05 in bold).

Chapter 5: Microsatellite analysis of albacore tuna (*Thunnus alalunga*): population genetic structure in the North East Atlantic and Mediterranean Sea

5.1. Abstract

Stock heterogeneity was investigated in albacore tuna (*Thunnus alalunga*, Bonnaterre, 1788), a commercially important species in the North Atlantic Ocean and Mediterranean Sea. Twelve polymorphic microsatellite loci were examined in 584 albacore tuna from eight locations, four in the North East Atlantic Ocean, two in the Mediterranean Sea and two in the South Western Pacific Ocean. Numbers of alleles per locus ranged from 9 to 38 (mean, 5.2 to 22.6 per locus; overall mean, 14.2 ± 0.47 S.E.) and observed heterozygosities per locus ranged from 0.44 to 1.00 (mean: 0.79 ± 0.19 S.E.). Significant deficits of heterozygotes were observed in 20% of tests, 7% of which were attributed to null alleles. Estimated null allele frequency ranged from 0.007 to 0.087. Low multilocus F_{ST} values were observed (-0.020 to 0.047 mean 0.016).

Pairwise F_{ST} values and exact tests showed that the South Pacific, North Atlantic and Mediterranean stocks were distinct from one another, thus corroborating findings in previous studies based on mitochondrial DNA, nuclear DNA (other than microsatellites) and allozyme analysis. Heterogeneity was observed for the first time between samples from the western and central Mediterranean Sea. There was evidence of temporal stability in samples collected in 2006 and 2007 from the central Mediterranean Sea. There was evidence for weak genetic structuring in the North East Atlantic with differences attributed to samples being collected either early or late in the fishing season. Observed genetic structure may be related to migration patterns and timing of movements of sub-populations to the feeding grounds in either summer or autumn. It is suggested that a more intensive survey is conducted throughout the entire fishing season to ratify or refute genetic homogeneity within the North East Atlantic albacore stock.

Keywords: Albacore tuna, *Thunnus alalunga*, microsatellites, population structure.

5.2. Introduction

Waldman (1999) defines a "stock" as an exploitable population with some degree of genetic integrity. There are also many definitions of stock, which have less or no emphasis on genetic structure (Cadrin *et al.*, 2005). Stocks can be delineated from observations relating to various aspects of life history (Griffiths, 1997). Discrimination of stock components into genetic stocks can be undertaken by molecular methods, such as allozymes analysis, mitochondrial DNA studies or by investigations into the variability within nuclear DNA (Ferguson, 1994). The stock structure of albacore has been identified globally by a variety of methods; primarily from information gathered directly from the fishery. Catch rates from each location and catch at length data, incorporated with information from ages determined from the calcareous structures, have been used to determine differences in growth rates and stock abundance in each ocean basin (ICCAT, 1996; Miyake *et al.*, 2004). In addition, conventional tag-recapture studies, using plastic floy tags attached to individual fish, have provided information on the migratory movements of albacore. It is considered that separate North and South stocks are present both in the Atlantic and Pacific Oceans as there is no evidence to date of cross equatorial migration from conventional tag-recapture studies and because of observed latitudinal differences in catch rates and seasonality of spawning (ICCAT, 1996; Ramon and Bailey, 1996). Therefore fish in the northern and southern hemispheres are managed as separate stocks. Although Beardsley (1969) proposed that small numbers may undertake inter-oceanic migrations between the South Atlantic Ocean and the Indian Ocean, this is yet to be substantiated through tagging studies and so the Indian Ocean population is managed as a separate stock (Chen *et al.*, 2005). Results from tagging surveys (Arrizabalaga *et al.*, 2002; Arrizabalaga *et al.*, 2003) have however shown that only very limited migration occurs between the North Atlantic Ocean and Mediterranean Sea and genetic differences have been observed between the two regions using nuclear DNA (Nakadate *et al.*, 2005). Consequently, the Mediterranean stock is managed as a separate unit (ICCAT, 1996). Local population differences have also been identified in the South Pacific using biological markers such as intestinal parasites which are endemic to certain oceanographic areas (Jones, 1991). In summary, based on information gathered from the fishery six populations of albacore

are recognised as stock units: Northern Atlantic, Southern Atlantic, Mediterranean, Indian, Northern Pacific and Southern Pacific (Miyake *et al.*, 2004; ICCAT, 2007).

Genetic markers can be used to assign individuals in mixed stock fisheries to the source populations (Shaklee *et al.*, 1999; Ruzzante *et al.*, 2004; Berg *et al.*, 2005). Stock identification by genetic methods may also indicate previously unidentified population structuring (Hoarau *et al.*, 2004; Carlsson *et al.*, 2006; Was *et al.*, 2008). Results from molecular genetic studies presently support the recognised subdivision of albacore populations into the six recognised stocks. Initially, no significant differences in mitochondrial DNA (mtDNA) were observed between albacore sampled in the South Atlantic and North Pacific Oceans (Graves and Dizon, 1989). In contrast, Chow and Ushiama (1995) using PCR-RFLP analysis of the mtDNA ATPase gene showed genetic differentiation between North and South Atlantic and Indo-Pacific albacore populations. An analysis of the mtDNA control D-loop region of albacore in the Indo-Atlantic region by Yeh *et al.* (1997) showed that populations in the South Atlantic and Eastern Indian Oceans were genetically distinct. Investigations into the genetic structure of North Atlantic and Mediterranean stocks, also using the mtDNA D-loop region (Viñas *et al.*, 1999) as well as allozymes (Pujolar *et al.*, 2003), showed genetic homogeneity between the two stocks. However, differences in morphometric characteristics, growth rates and reproductive areas had been previously reported for the two stocks (Megalofonou, 2000), Viñas *et al.* (2004) conducted an additional study using the mtDNA control region in combination with nuclear DNA markers, and their results showed that there was a small but significant difference between the two stocks. Nakadate *et al.* (2005) using nucleotide sequence variations of the glucose-6-phosphate dehydrogenase gene intron (G6PDH) and the mtDNA D-loop region corroborated their findings. Analysis of blood lectins (Arrizabalaga *et al.*, 2004) indicated that the North East Atlantic, South Atlantic and South East Pacific populations were distinct but that South Atlantic and Indian Ocean populations were genetically similar.

Many of the previous studies address differences between stocks in different oceanic regions, with few investigating genetic heterogeneity within regions. Recently, Wu *et al.* (2009) studied albacore from three areas in the Northwestern Pacific Ocean (Taiwan, Japan and North of Hawaii). Analysis of mtDNA sequence data showed that albacore tuna in this region constituted a single stock with no significant differences in geographic distributions. In another study based on

allozymes, Hue (1979; 1980) suggested that there were two separate sub-populations of albacore within the North Atlantic region, the "Classic" and "Azores" populations. These proposed sub-populations are distinguished by migration pathways. This separation appears to occur at 18°W with the "Classic" population migrating to the east of 18°W closer to Europe and northern Africa, while the "Azores" component is mainly found to the west of 18°W near the Azores Islands in the summer months before moving towards the central North Atlantic to winter feeding grounds. To date, there have been no further studies on albacore in this region.

Advances in molecular techniques have led to the development of effective tools, such as microsatellite loci, for identifying genetic heterogeneity on a finer geographic scale in the more intensively studied bluefin tuna (*Thunnus thynnus*) (Broughton and Gold, 1997; Carlsson *et al.*, 2004; Carlsson *et al.*, 2006), and have the potential to offer insights into stock structure of albacore. In recent years, highly variable genetic markers such as microsatellite loci have been successfully employed in the investigation of structuring in tuna stocks and have revealed significant genetic heterogeneity in a range of tuna species in the North Atlantic, Indian and South Pacific Oceans (Appleyard *et al.*, 2001; Carlsson *et al.*, 2006; Díaz-Jaimes and Uribe-Alcocer, 2006; Dammannagoda *et al.*, 2008). Microsatellite loci consist of short repeat sequences (two to five base pairs), and are distributed throughout most eukaryotic genomes (O'Connell and Wright, 1997). The advantages of using microsatellites in population studies is that these sequences undergo comparatively rapid mutation rates that can lead to high levels of polymorphism, and this makes them ideal for detecting differences among closely related populations (O'Connell and Wright, 1997). There are also disadvantages to using these markers, microsatellite loci can be subject to high levels of null alleles (Paetkau and Strobeck, 1995; Chapuis and Estoup, 2007; Falush *et al.*, 2007), which can lead to scoring errors and thus compromise the validity of the resulting data, although attempts can be made to correct for these errors (Van Oosterhout *et al.*, 2004; Chapuis and Estoup, 2007). In spite of potential disadvantages, this class of markers tend to be preferred to previous markers that are characterised by lower levels of polymorphism, such as nuclear-encoded proteins (Edmunds and Sammons, 1973; Thompson and Contin, 1980), mtDNA cyt*b* sequence data (Bartlett and Davidson, 1991) and allozymes (enzymes) analysis (Pujolar *et al.*, 2003) which failed to detect population structure in the Atlantic Ocean. For albacore tuna, a preliminary study using microsatellite loci

revealed more marked levels of differentiation between and within Atlantic and Pacific Oceans compared to mtDNA analyses of samples from the same areas (Takagi *et al.*, 2001), further indicating that this class of markers appears to be more powerful in detecting subtle population structure of albacore tuna. With the exception of Takagi *et al.* (2001), no microsatellite studies have been conducted on albacore tuna.

In view of the paucity of information on the genetic structure of albacore tuna in the Northeast Atlantic and Mediterranean Sea, the main objective of the present study was to analyse spatial, seasonal and temporal genetic heterogeneity using 12 microsatellite markers in albacore tuna collected in consecutive years from 2005 to 2007, from four North East Atlantic areas (waters off the south west of Ireland, towards the southern Bay of Biscay along the Porcupine Ridge and off the northern coast of Africa near the Canary Islands), and from central (Tyrrhenian Sea) and west (Balearic Sea) Mediterranean Sea regions. All albacore sampled with the exception of those collected from near the Canary Islands were juveniles.

5.3. Materials and Methods

Sampling

A total of 14 samples (N=584) of albacore were collected from the North East Atlantic Ocean (SW Ireland, South Bay of Biscay and Canary Islands) and Mediterranean Sea using a variety of fishing methods (Figure 5.1). All samples were obtained from commercial fishing operations, which targeted aggregations of fish in summer feeding grounds. Fish were measured for fork length (L_F) to the nearest centimetre, weighed to the nearest 10g and sexed by visual inspection of the gonad. Maturity was assigned on the basis of age and length, fish less than 5 years and with a $L_F < 90$cm were considered immature or juveniles (Santiago and Arrizabalaga, 2005). Details of sampling are shown in Table 5.1. A $5mm^3$ piece of white muscle was removed from behind the head in each individual and stored in 96% ethanol. Two South-West Pacific Ocean samples from archived freeze-dried tissue (2003 and 2005) were acquired to serve as out-groups.

Microsatellite analysis

Genomic DNA was extracted from muscle tissue (~$2mm^3$) using the phenol-chloroform method of Sambrook *et al.* (1989). DNA was diluted 1:5 in sterile deionised water to give a concentration of 30-100ng μl^{-1}. Twelve microsatellite loci developed for bluefin tuna, which cross-amplified in albacore, were selected for analysis (see Chapter 4): *Ttho*4, *Ttho*6, *Ttho*7 (Takagi *et al.*, 1999), *Tth*5 (McDowell *et al.*, 2002), *Tth*4, *Tth*14, *Tth*17, *Tth*185, *Tth*254, *Tth*1-31, *Tth*12-29 and *Tth*16-2 (Clark *et al.*, 2004). The reverse primer of each pair was end-labelled with fluorescent dye (700-IRD or 800-IRD, Li-COR, Lincoln, NE, USA). Polymerase chain reaction (PCR) was carried out using a reaction volume of 10µl, containing 0.17U *Taq* polymerase, 1x reaction buffer (Bioline), 0.25 µM of each primer, 2µl of mixed dNTPs, 0.2 mM $MgCl_2$ and 1µl of the 1.5 dilution of template DNA. Thermocycling procedures for each locus were exactly those in Takagi *et al.* (1999), McDowell *et al.* (2002) and Clark *et al.* (2004).

Amplification products were separated on 6% polyacrylamide gels using a Li-COR 4300 automated sequencer (Li-COR, Lincoln, NE, USA). PCR products were

diluted 1:5–1:15 with deionised water, and 1µl of the dilution mixed 1:3 with bromophenol blue in formamide loading buffer. A sizing standard (50-350 base pairs, Li-COR, Lincoln, NE, USA) was run in the centre and at both ends of the gels to calibrate allele size. An internal reference sample consisting of individuals where allele sizes had been pre-determined was included to ensure consistency in genotype scoring across runs. Fragment length polymorphisms were scored with GENE IMAGIR software (Li-COR, Lincoln, NE, USA).

Data Analysis

Allelic distribution, observed (H_O) and unbiased expected (H_E) heterozygosity estimates for the 14 samples were computed for each locus individually and as a multilocus estimate using GENETIX 4.05.2 (Belkhir *et al.*, 2002). Tests for conformance to Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium between pairs of loci, were performed using GENEPOP 3.4 (Raymond and Rousset, 1995) with specified Markov chain parameters (10000 dememorization steps, 100 batches, 5000 iterations per batch). Single and multilocus F_{IS} (indicating heterozygote deficiency/excess) were estimated (Weir and Cockerham, 1984) and significance was tested using GENEPOP 3.4 (Raymond and Rousset, 1995). The possible presence of genotyping errors due to null alleles, large allele drop-out or stuttering was tested using MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.*, 2004). Possible genotyping errors were corrected for by the Oosterhout null allele estimator in MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.*, 2004). This method assumes that there are no null allele homozygotes and ignores all non-amplified samples as degraded DNA, human error etc. Population differentiation was analysed using pairwise F_{ST} estimates and their significance was tested by permutation tests (1000) using GENETIX 4.05.2 (Belkhir *et al.*, 1996). Pairwise exact tests were performed in GENEPOP 3.4 (Raymond and Rousset, 1995). The false discovery rate of Benjamini and Yekutieli (2001) was applied to correct for multiple pairwise comparisons. This method accommodates large number of potentially dependant tests while balancing risks of Type I and Type II error and is a good alternative to the very conservative Bonferroni correction (Rice, 1989), which is effective in reducing Type I, but not Type II errors (Narum, 2006). Bootstrap values were calculated in GENETIX 4.05.2 (Belkhir *et al.*, 1996). A multi dimensional scaling plot was created in SPSS version

15 (SPSS, 2006) to compare the relationship in space of one sample versus the other between the pairwise F_{ST} values.

5.4. Results

Genetic diversity and HWE

A total of 584 fish from 14 locations in the NE Atlantic Ocean, Pacific Ocean and Mediterranean Sea were genotyped at 12 microsatellite loci. All loci were highly polymorphic with a maximum of 9 to 38 alleles per locus (mean 5.2 to 22.6) with a similar level of polymorphism across samples (Table 5.2). Of the 301 alleles detected at the 12 loci, 43 were private i.e. only occurring within a single sample, with frequencies no higher than 0.006. Observed heterozygosity values ranged from 0.44 for *Tth*16-2 to 1.00 for *Tth*4. Mean multilocus observed heterozygosities were similar across all samples (0.74-0.82; Table 5.2). Significant departures (heterozygote deficits) from HWE were observed for all loci and in all samples; 18/168 and 16/168 tests were significant at $P<0.05$ and $P<0.01$, respectively (Table 5.2). Multilocus F_{IS} estimates were significant in 8 of the 14 samples.

MICROCHECKER (Van Oosterhout *et al.*, 2004) analysis indicated that null alleles were present for six of the 12 loci, *Ttho*6, *Tth*14, *Tth*254, *Tth*1-31, *Tth*12-29 and *Tth*16-2, in between one and four of the fourteen samples tested. The frequencies of null alleles was calculated (Table 5.3) using the Oosterhout null allele estimator (Van Oosterhout *et al.*, 2004). As frequencies of null alleles were low, (0.007-0.104) and were not consistent across samples at a particular locus, it was decided to include all 12 loci in further data analysis after data had been corrected for null alleles using MICROCHECKER.

There was some indication of possible linkage disequilibrium in pairwise tests; these were between *Ttho*4 and *Ttho*7; *Ttho*6 and *Tth*4, *Tth*254, *Tth*1-31; *Tth*17 and *Ttho*7, *Tth*4, *Tth*185, *Tth*16-2; *Tth*254 and *Tth*4, *Tth*5; but these were not consistent across samples or loci. Therefore, it was concluded that no evidence of strong physical linkage was found between all pairs of loci tested among all sampled areas.

Population differentiation between regions

Results from pairwise multilocus F_{ST} estimates, and exact tests of genic and genotypic proportions indicated that the two samples from the South West Pacific

(2003 and 2005) were genetically similar but differed significantly from all samples from the Mediterranean Sea and NE Atlantic Ocean (Table 5.4). These findings were corroborated using exact tests (Table 5.4).

All three Mediterranean Sea samples were shown to be genetically distinct from all of the North Atlantic Ocean samples (Table 5.4) from pairwise F_{ST} estimates and exact tests. High bootstrap support was evident for the pairwise F_{ST} estimates, ranging from 0.010 - 0.029. The difference between F_{ST} values is clearly illustrated in the MDS plot (Figure 5.2), where samples from the three regions are clearly separated.

Population differentiation within regions

Of 36 pairwise F_{ST} estimates for the North East Atlantic, 16 were significant (Table 5.4). A higher number of pairwise exact tests were significant, 22 of 36 in the North East Atlantic (Table 5.4). The pairwise F_{ST} values and exact tests in the North East Atlantic samples showed genetic homogeneity between six of the nine samples (NEAtl05_1, NEAtl05_3, NEAtl06_3, NEAtl07_1, NEAtl07_2 and NEAtl07_3; Table 5.4). These samples were obtained from feeding grounds covering approximately 2,500 kilometres from southwest Ireland to the southern Bay of Biscay (Figure 5.1). There was evidence for genetic heterogeneity in pairwise comparisons involving the three samples: NEAtl05_2, NEAtl06_1 and NEAtl06_2 (Table 5.4). All of the pairwise comparisons involving the NEAt06_1 sample were significant, indicating that this sample is differentiated from all other samples from the North East Atlantic. Six F_{ST} estimates and seven exact tests of the eight pairwise comparisons involving sample NEAtl06_2 were significant, indicating a high degree of population differentiation. Half of the pairwise F_{ST} comparisons and six of eight pairwise exact tests involving NEAtl05_2 were significant (Table 5.4). These three samples (NEAtl05_2, NEAtl06_1 and NEAtl06_2) were caught early in the fishing season (July and August) (Table 5.1), whilst the other five juvenile North Atlantic samples, were caught between the end of September and October (Tables 5.1 and 5.4). The MDS plot (Figure 5.2) indicates little evidence of sub-clustering within the North Atlantic samples with the exception of NEAtl06_1.

Five of eight juvenile samples (NEAtl05_1, NEAtl05_2, NEAtl06_3, NEAtl07_1 and NEAtl07_2) from the North East Atlantic were genetically similar to

the adults collected from the Canary Islands (NEAtl07_3); i.e. there were no significant differences in pairwise comparisons of F_{ST} values and exact tests (P>0.05) for these five samples (Table 5.4). The samples of juvenile albacores caught in the early fishing season in 2006 (NEAtl06_1 and NEAtl06_2; Table 5.1) showed significant differences in F_{ST} values and exact tests (P<0.05) when compared with the sample of adult albacores from the Canary Islands (NEAtl07_3) (Table 5.4).

When the three Mediterranean samples were compared, the two samples from the central region of the Mediterranean Sea did not differ significantly from each other, indicating an absence of temporal heterogeneity between the 2006 and 2007 samples. Pairwise F_{ST} estimates showed that the samples collected from the western side of the Mediterranean Sea in 2005 (Med05), differed significantly from the two samples from the central region (Med06 and Med07). However, results, from exact tests indicated significant heterogeneity between all three samples (Table 5.4). The two samples from the Pacific Ocean did not differ for either F_{ST} or exact test pairwise comparisons. In summary, significant heterogeneity was observed between samples from the Mediterranean Sea, there is also evidence of genetic structure within the albacore feeding aggregations in the NE Atlantic Ocean along the Porcupine ridge.

5.5. Discussion

Genetic diversity and HWE

The present study used microsatellite markers to investigate the genetic structure of albacore tuna both within and between different oceanic regions. Twelve microsatellites were screened, all of which were developed for bluefin tuna (Takagi *et al.*, 1999; McDowell *et al.*, 2002; Clark *et al.*, 2004). Three of the microsatellites used in the present study had previously been utilized by Takagi *et al.* (2001) to evaluate genetic variation within and between albacore samples from the North and South, Atlantic and Pacific Oceans. Similar numbers of alleles were observed in the two studies at loci *Ttho*6 (N_A (this study) = 18 and N_A = 19 (Takagi *et al.*, 2001)). At *Ttho*-4 and *Ttho*7 in NE Atlantic samples, there were approximately twice as many alleles observed in this study (Mean N_A = 32 and 26, respectively) compared to Takagi *et al.* (2001) (N_A =11 and 12, respectively). Mean heterozygosities per locus were similar in both studies, for all loci and areas sampled; heterozygosities were similar in range to studies on other species of tuna (Appleyard *et al.*, 2001; Carlsson *et al.*, 2004).

Significant departures from HWE were observed at all loci and in all samples (Table 5.2). Deviations from HWE caused by a deficit of heterozygotes is well documented in marine fish (O'Connell and Wright, 1997). Although factors such as inbreeding, the Wahlund effect, or selection can explain such deficits, they are very often an artifact of the PCR amplification process (O'Connell and Wright, 1997). For example, deficits can arise when alleles (referred to as null alleles) fail to amplify because of base substitutions or deletions in PCR priming sites flanking microsatellite arrays. Null alleles are a common problem with microsatellite loci, and can lead to high, observed deficits of heterozygotes (Dakin and Avise, 2004). Deficits may also be due to preferential amplification of small alleles (Wattier *et al.*, 1998) or slippage during PCR amplification (Shinde *et al.*, 2003). MICRO-CHECKER was used to test for these three scenarios and it was found that 12 of the 34 significant departures from HWE were attributable to null alleles (Table 5.2). Null alleles may cause a reduction in genetic diversity within populations (Paetkau and Strobeck, 1995; Chapuis and Estoup, 2007), and consequently affect the estimation of population differentiation. In other words F_{ST} values and genetic distance generally increase with decreasing

within-population genetic diversity. Null alleles in microsatellites have been observed in other tuna species (Clark *et al.*, 2004). Because of this, data for population differentiation analysis were corrected for null alleles (Van Oosterhout *et al.*, 2004).

Population differentiation between regions

Multilocus pairwise comparisons of F_{ST} values were low, ranging from -0.020 to 0.047 with an average value of 0.034. Despite relatively low F_{ST} values, 69 of 91 (76%) comparisons were significant in this study. There were a greater number of significant values from exact tests of genic proportions (76 of 91, 84%) which reflects the enhanced power of exact tests to detect significant population structuring (Balloux and Lugon-Moulin, 2002). Overall, results from pairwise F_{ST} estimates and exact tests, and the MDS plot (Table 5.4 and Figure 5.2) indicate that North Atlantic Ocean, Mediterranean Sea and South Pacific are strongly differentiated from one another. The finding of significant genetic differentiation between North Atlantic and southwest Pacific albacore corroborates those of Chow and Ushiama (1995), where haplotype analysis of the mitochondrial ATPase gene indicated genetic heterogeneity between the Atlantic and Pacific stocks, but showed homogeneity within both stocks. In addition, the genetic differentiation between North Atlantic Ocean and Mediterranean Sea albacore found in this study was also reported by Arrizabalaga *et al.* (2004), Viñas *et al.* (2004) and Nakadate *et al.* (2005) for a variety of markers, such as blood lectins, mtDNA and nuclear markers. All these studies validate non-molecular differences reported between North Atlantic and Mediterranean albacore (Megalofonou, 2000). There is now ample genetic evidence to support the justification of managing albacore in the Mediterranean Sea as a separate entity to albacore in the North Atlantic Ocean.

The science of landscape ecology is increasingly being combined with population genetics to explain differentiation between populations of a species (Manel *et al.*, 2003). Isolation by distance and physical barriers to gene flow are two factors often proposed to explain differences within species across different geographic areas, for example, as the basis for the separation of bluefin tuna into two sub-species, one which inhabits the Atlantic Ocean (*Thunnus thynnus thynnus*) and the other the Pacific Ocean (*T. t. orientalis*) (Ward, 1995). Landscape genetics can be

applied to marine studies where both visible and invisible oceanographic features, such as benthic topography and currents, can lead to the segregation of marine populations with pelagic stages in the life history (Jørgensen *et al.*, 2005; Karlsson and Mork, 2005; Was *et al.*, 2008). The observed genetic distinctness of North Atlantic Ocean and Mediterranean Sea albacore is particularly interesting in the light of the geological separation of the two regions in the late Miocene period (~ 5.9 million years ago) and reconnection during the Pliocenic period of the late Cenozoic period, some 5.33 million years ago (Patarnello *et al.*, 2007). The Mediterranean Sea is a fully-enclosed sea except for the narrow and deep connection with the North Atlantic Ocean, and the majority of biota have colonized the Mediterranean Sea from the Atlantic Ocean through this entrance, the Straits of Gibraltar (Figure 5.1) (Almada *et al.*, 2001; Domingues *et al.*, 2005). There are conflicting opinions as to whether the Straits of Gibraltar act as a barrier to gene flow, some studies indicate a transition zone of allele frequencies that is more closely associated with the Almeria-Oran front (Figure 5.1) (Cimmaruta *et al.*, 2005). Patarnello *et al.* (2007) reviewed over 70 studies on Atlantic-Mediterranean species differentiation and structuring was observed in 41 of these studies. Three possible hypotheses for phylogeographic structuring were proposed, (i) full congruence/panmixia, suggesting that there is high gene flow between both seas, as shown in European pilchard (*Sardina pilchardus*), chub mackerel (*Scomber japonicus*) and seabream (*Diplodus sargus*); (ii) distinct Atlantic and Mediterranean clades caused by historic separation, recent isolation by distance and distinct population dynamics, such as in sea bass (*Dicentrarchus labrax*), anchovy (*Engraulis encrasicolus*), hake (*Merluccius merluccius*), and Sand goby (*Pomatoschistus minutus*); (iii) patterns which differ from hypotheses i and ii, such as in Atlantic mackerel, *Scomber sombrus*, where the East Mediterranean clade is distinct from the West Mediterranean and Atlantic Ocean (Patarnello *et al.*, 2007). Patarnello *et al.* (2007) concluded that there no uniform phylogenetic patterns could be applied to all fish species inhabiting the Atlantic and Mediterranean Seas. In this study clear separation was evident between albacore from the North Atlantic and Mediterranean, which conforms to hypothesis ii of Patarnello *et al.* (2007). Albacore are similar in morphology (Pujolar *et al.*, 2003) and environmental preference (Beardsley, 1969; Chow and Kishino, 1995) to bluefin tuna (*T. t. thynnus*) and both are considered part of the "bluefin" tuna group that occupy cooler waters, yet bluefin tuna migrate out of the Mediterranean Sea through the straights of Gibraltar (Carlsson

et al., 2006) whereas for albacore Atlantic-Mediterranean migrations have been shown to be limited (Arrizabalaga *et al.*, 2002; Arrizabalaga *et al.*, 2003). The comparative differences in behaviour of these two species raises questions as to why limited Atlantic-Mediterranean movement is observed in albacore when these fish, as a fast moving pelagic species tolerant of cooler waters, have the physiology to cross oceanographic features such as the Almeria-Oran front and the Straits of Gibraltar.

Population differentiation within regions

Albacore in the North Atlantic Ocean is currently managed as a single stock and no genetic structuring is recognised within the population (ICCAT, 2007) in spite of studies that indicate possible structuring (Hue, 1979; 1980; Arrizabalaga *et al.*, 2004). The results of this study indicate that there may be both spatial and seasonal structuring within the North Atlantic Ocean. Pairwise F_{ST} and exact tests of data corrected for the presence of null alleles indicates that samples NEAtl06_1 and NEAtl05_2 (from Goban Spur along the Porcupine ridge) and NEAtl06_2 (from southern Bay of Biscay) are the most genetically distinct from other NE Atlantic samples collected in 2005 and 2007; however, the MDS plot indicates that NEAtl05_2 and NEAtl06_2 cluster with the other North East Atlantic samples. Variation between the samples obtained along the Porcupine ridge in the North Atlantic may occur because fish from more than one stock migrate to the feeding areas along different migration pathways. Hue (1979; 1980) proposed that North Atlantic albacore are differentiated into at least two sub-populations with separate seasonally distinct migration routes (i.e. the "Classic" and the "Azores"); the fish that follow these separate migration routes can be characterized by observed differences in morphometrics (head length vs. body length) and by the analysis of proteins from the eye lenses. The results in this study may indicate seasonal partitioning within a fishing season, as separate populations migrate to the feeding areas at different times of the year. The samples showing most differences in pairwise comparisons were from albacores caught in the early fishing season (July and August) (Table 5.1 and 5.4). Two of these samples also showed differences when compared to the adult sample from the Canary Islands (NEAtl07_3); this indicates that it is possible that these fish do not recruit to a singe adult population. Although caution is required when interpreting these results as the Canary Island sample is based on a smaller

number of individuals (N= 20) than that recommended for microsatellite population analysis (i.e. a minimum of N = 50) (O'Connell and Wright, 1997). In a similar study on bluefin tuna Carlsson *et al.* (2006) was able to determine whether individual bluefin tuna in a feeding aggregation in the North Atlantic belonged to either the Eastern or Western stock; these two stocks have been shown to migrate to feeding grounds at different times but are present for a few months as a mixed stock. The structuring of albacore as a transient population in the North East Atlantic may be based on different timing of migration to feeding areas, and the observation of genetic structuring may be dependant on the month the samples are collected during the fishing season. Further information needs to be gathered to track the movement of the different components migrating into the feeding assemblages as well as collecting adult albacores from spawning grounds. Data on intra-oceanic migration pathways may be ascertained from archival tags, such as those used on larger fish (Sims *et al.*, 2003). A further study including more intensive sampling throughout the fishing season would be needed to confirm or disprove the suggested structuring where different populations may be migrating to the feeding areas at different times. The combination of investigating migration pathway and timings with microsatellite data may provide further information in order to either refute or ratify genetic homogeneity within the North Atlantic stock.

Megalofonou (1990) and Cefali *et al.* (1986) cited in Megalofonou (2000) have shown that the distribution of albacore in the Mediterranean is discontinuous, with the highest concentrations found in the Tyrrhenian Sea in the Western Mediterranean Basin (Figure 5.1; location of samples Med06 and Med07) and the Ionian, Adriatic and Aegean Seas in the Eastern Mediterranean Basin (not shown). Previous studies have shown that oceanographic barriers appear to exist within the Mediterranean Sea, most notably the Almeria-Oran and the Siculo-Tunisian fronts (Figure 5.1), which separate the Mediterranean into the East and West basins. Carlsson *et al.* (2004) proposed possible heterogeneity of bluefin tuna within the Mediterranean, with the distinction being most evident between the Tyrrhenian and Ionian Seas, i.e. between the east and west basin separated by the Siculo-Tunisian front. Genetic heterogeneity between the East and West basins has been observed in other species, from those with sedentary and slow dispersal (sea grass, *Posidonia oceanica* (Arnaud-Haond *et al.*, 2007) and cuttlefish, *Sepia officinalis* (Pérez-Losada *et al.*, 2007)) to mobile species such as sea bass, *Dicentrarchus labrax* (Bahri-Sfar *et*

al., 2000) and anchovy, *Engraulis encrasicolus* (Magoulas et al., 2006). In this study it was found that there was little difference in F_{ST} values and exact tests between Med 2006 and 2007 samples from the Tyrrhenian Sea indicating temporal stability in genetic structure. However, large differences were observed between samples from the Balearic Sea (Med05) and the Tyrrhenian Sea (Med06 and Med07), indicating possible heterogeneity within albacore in the western Mediterranean basin. This finding has not been reported in other studies on tuna. It is therefore possible that additional heterogeneity to that observed in the present study may exist in albacore within the Mediterranean Sea. Unfortunately, it was not possible to obtain samples from the Eastern Mediterranean basin (Adriatic, Ionian and Aegean Seas) for this investigation.

In conclusion, significant population structuring was observed in both North Atlantic and Mediterranean albacore, despite potentially high gene flow by larval drift, high fecundity, large population size (ICCAT, 2007), large catch sizes, and the extensive trans-oceanic feeding and spawning migrations that take place. It is proposed that further work should be undertaken to include a greater number of samples from a wider geographic area and more frequently across the four seasons within each year. This further work should include samples from spawning aggregations in the Eastern Atlantic Ocean (Beardsley, 1969) and throughout the entire Southern Mediterranean Sea (Piccinetti and Piccinetti Manfrin, 1993). At present the North Atlantic albacore stock is managed as a single unit, with the Mediterranean stock as a separate entity (ICCAT, 2007). If heterogeneity exists within both stocks on the basis of different migration patterns, discontinuous distribution, morphometric traits and molecular data, then this may have implications for stock management if one sub-population contributes more to the effective population size than another. Bias in stock assessment could lead to the possible elimination of some sub-populations (Carvalho and Hauser, 1994) by overfishing of recruits or spawning stock.

Table 5.1. Details of albacore tuna (*Thunnus alalunga*) samples. Location abbreviations as in Figure 1; - indicates no information/data; fishing method L = pelagic longline, P = pair trawling, T = trolling; maturity based on size J = juvenile, A = adult.

Sample	Sample Size	Location	Date	Survey Vessel	Fishing Method	Mean LF (cm)	Mean Weight (kg)	% Female	Maturity	Age (years)/number of fish
Med05	52	40 00'N 1 34'E	Nov 2005	-	L	63 ± 2. 6	4.68 ± 0.61	-	J	3/22, 4/28, 5/2
Med06	50	39 49'N 13 00'E	Nov 2006	-	L	65 ± 4.0	5.06 ± 0.97	42	J	3/19, 4/17, 5/10, 6/1 (no information for 3 fish)
Med07	50	39 49'N 13 00'E	Dec 2007	-	L	75 ± 3.4	7.90 ± 1.10	16	J	2/1, 3/8, 4/22, 5/16, 6/3
NEAtl05_1	38	48 30'N 10 38'W	Sept 2005	MFV Maggie C	T	56 ± 5.7	-	-	J	2/31, 3/5, 4/0, 5/1, 6/2
NEAtl05_2	50	47 34'N 12 28'W	July 2005	MFV Mellifont	T	62 ± 7.9	5.62 ± 2.07	-	J	2/23, 3/37, 4/9, 5/2
NEAtl05_3	50	48 21'N 10 29'W	Sept 2005	MFV Supreme 2	P	66 ± 10.2	-	-	J	2/13, 3/17, 4/18, 5/2
NEAtl06_1	57	47 32'N 9 30'W	Aug 2006	MFV OceanDawn	T	59.5 ± 8.5	5.00 ± 2.60	-	J	2/35, 3/11, 4/7, 5/2 (no information for 1 fish)
NEAtl06_2	67	44 50'N 3 20'W	Aug 2006	MFV Skipper	P	74 ± 12.1	9.60 ± 4.50	-	J	2/13, 3/10, 4/17, 5/22, 6/3, 7/1, 8/1
NEAtl06_3	16	43 36'N 2 23'W	Sept 2006	-	T	54 ± 9.9	1.40 ± 0.20	-	J	1/6, 2/8, 3/2
NEAtl07_1	46	52 19'N 12 23'W	Sept 2007	MFV DeLinn	T	72 ± 4.4	8.03 ± 1.69	-	J	3/7, 4/24, 5/11, 6/3 (no information for 1 fish)
NEAtl07_2	50	51 33'N 13 51' W	Sept 2007	MFV TeresaMae	T	67 ± 5.0	-	-	J	2/1, 3/30, 4/17, 5/2
NEAtl07_3	20	27 00'N 17 00'W	Mar 2007	-	-	106.8 ± 7.6	-	-	A	-
Pac03	18	21 00'S 163 50'E	July 2003	-	-	89 ± 4.6	-	42	A	-
Pac05	20	14 00'S 135 00'W	Sept 2005	-	-	98 ± 4.6	-	25	A	-

Table 5.2. Summary of genetic variation in albacore at twelve microsatellite loci at 14 locations. N = number of individuals, N_A = number of alleles per locus, H_E = unbiased expected heterozygosity (gene diversity), H_O = observed heterozygosity, F_{IS} = inbreeding coefficient (Weir and Cockerham, 1984), ♦ denotes presence of null alleles (Van Oosterhout, 2004). The right column gives the mean numbers of alleles, N_A, over the total number of alleles observed, together with mean F_{IS} value, for that locus. P<0.05 emboldened, P<0.01 values with asterisks.

Locus/Sample	Med05	Med06	Med07	NEAtl05_1	NEAtl05_2	NEAtl05_3	NEAtl06_1	NEAtl06_2	NEAtl06_3	NEAtl07_1	NEAtl07_2	NEAtl07_3	Pac03	Pac05	Mean N_A/locus
	N 52 ±1	N 50 ±1	N 50 ±2	N 38 ±2	N 50 ±1	N 50 ±2	N 57 ±2	N67 ±0	N 16 ±1	N 46 ±1	N 50 ±1	N 20 ±4	N 20 ±1	N 18 ±0	
Ttho4															
N_A	21	21	21	19	21	21	21	20	17	19	26	14	18	15	19.57
H_E	0.91	0.91	0.88	0.90	0.91	0.87	0.90	0.91	0.92	0.86	0.90	0.85	0.90	0.89	
H_O	0.83	0.86	0.88	0.92	0.94	0.90	0.84	0.91	0.88	0.87	0.86	0.85	0.85	1.00	
F_{IS}	**0.091***	0.056	0.000	-0.021	-0.037	-0.033	0.062	-0.006	0.046	-0.015	0.042	0.005	0.053	-0.131	0.002
Ttho6															
N_A	9	9	7	11	11	10	13	11	8	10	11	7	7	9	9.50
H_E	0.73	0.75	0.71	0.71	0.73	0.78	0.75	0.70	0.72	0.77	0.71	0.53	0.67	0.72	
H_O	0.67	0.72	0.72	0.58	0.72	0.72	0.65	0.63	0.63	0.80	0.58	0.58	0.70	0.56	
F_{IS}	0.085	0.036	-0.014	**0.182***	0.015	**0.081**	**0.135♦**	0.106	0.140	-0.048	**0.184*♦**	-0.103	-0.041	**0.234***	0.062
Ttho7															
N_A	11	9	11	15	16	14	14	19	9	17	17	14	15	17	14.14
H_E	0.83	0.78	0.76	0.84	0.89	0.81	0.85	0.88	0.87	0.84	0.89	0.86	0.90	0.92	
H_O	0.88	0.82	0.75	0.82	0.86	0.80	0.82	0.88	0.81	0.76	0.94	0.71	0.90	0.83	
F_{IS}	-0.072	-0.046	0.012	0.025	0.030	0.014	0.031	**-0.006**	0.069	0.101	-0.055	0.181	0.000	0.093	0.027
Tth4															
N_A	25	22	21	27	27	24	26	28	15	25	29	17	16	15	22.64
H_E	0.92	0.91	0.93	0.96	0.94	0.95	0.94	0.95	0.95	0.95	0.95	0.95	0.94	0.94	
H_O	0.88	0.92	0.88	0.97	0.94	0.88	0.96	0.96	1.00	0.89	0.88	1.00	0.90	0.83	
F_{IS}	0.042	0.011	0.056	-0.015	0.005	**0.078**	-0.026	-0.004	-0.060	**0.067**	0.072	-0.054	0.041	**0.113**	0.023
Tth5															
N_A	16	17	16	18	21	21	20	21	15	20	19	13	16	15	17.71
H_E	0.88	0.90	0.88	0.90	0.93	0.92	0.90	0.92	0.92	0.92	0.89	0.91	0.91	0.93	
H_O	0.88	0.84	0.92	0.84	0.92	0.83	0.93	0.94	0.94	0.87	0.88	1.00	0.85	0.94	
F_{IS}	-0.006	0.072	-0.041	0.695	0.012	0.094	-0.027	-0.025	-0.025	0.058	0.013	-0.099	0.064	-0.011	0.055

*Tth*14								
N_A	7	5	6	5	7	6	6	4
H_E	0.64	0.60	0.63	0.58	0.62	0.62	0.69	0.62
H_O	0.52	0.60	0.62	0.71	0.58	0.64	0.56	0.66
F_{IS}	**0.193*♦**	0.006	0.010	-0.230	0.064	-0.029	**0.188*♦**	-0.067
*Tth*17								
N_A	17	18	17	19	21	23	23	20
H_E	0.84	0.92	0.92	0.93	0.92	0.90	0.94	0.92
H_O	0.85	0.86	0.86	0.84	0.86	0.83	0.88	0.90
F_{IS}	-0.011	0.062	**0.064***	**0.099**	0.063	**0.080**	**0.065**	**0.024***
*Tth*185								
N_A	18	17	16	19	21	22	23	22
H_E	0.88	0.90	0.86	0.93	0.94	0.93	0.92	0.93
H_O	0.83	0.92	0.90	0.86	0.96	0.84	0.89	0.87
F_{IS}	0.064	-0.022	-0.041	0.077	-0.017	0.098	0.027	0.072
*Tth*254								
N_A	16	17	23	23	18	25	20	27
H_E	0.90	0.90	0.92	0.94	0.91	0.94	0.92	0.94
H_O	0.71	0.78	0.96	1.00	0.80	0.98	0.82	0.81
F_{IS}	**0.210*♦**	**0.131♦**	-0.046	-0.068	**0.118♦**	-0.046	**0.100***	**0.139*♦**
*Tth*1-31								
N_A	11	16	13	9	11	12	13	17
H_E	0.81	0.81	0.78	0.77	0.81	0.79	0.84	0.85
H_O	0.67	0.74	0.86	0.87	0.86	0.80	0.86	0.87
F_{IS}	**0.175♦**	**0.090***	-0.100	-0.132	-0.060	-0.012	-0.024	**-0.018***
*Tth*12-29								
N_A	6	8	9	7	8	7	8	7
H_E	0.62	0.70	0.70	0.63	0.65	0.61	0.66	0.57
H_O	0.60	0.72	0.66	0.63	0.62	0.72	0.75	0.48
F_{IS}	0.034	**-0.025**	0.062	0.002	0.044	-0.189	-0.151	**0.170**

5	4	5	4	4	5	5.21
0.66	0.59	0.61	0.56	0.56	0.63	
0.81	0.52	0.66	0.65	0.60	0.92	
-0.250	0.119	-0.083	-0.165	-0.078	-0.151	-0.034
14	24	23	17	14	16	19.00
0.92	0.94	0.94	0.94	0.86	0.92	
0.94	0.91	0.88	0.94	0.90	0.94	
-0.025	0.034	**0.063**	-0.009	-0.049	-0.027	0.031
14	21	20	12	17	18	18.57
0.94	0.93	0.92	0.93	0.94	0.95	
0.88	0.91	0.88	0.94	1.00	1.00	
0.067	0.021	0.048	-0.016	-0.064	-0.057	0.018
18	24	23	15	15	16	20.00
0.94	0.93	0.94	0.92	0.93	0.94	
0.94	0.89	0.92	1.00	0.95	0.89	
0.002	0.042	0.018	-0.088	-0.018	0.052	0.039
10	13	13	8	9	10	11.79
0.79	0.80	0.83	0.83	0.78	0.86	
0.69	0.83	0.94	0.82	0.85	0.94	
0.134	-0.030	-0.140	0.011	-0.091	-0.101	-0.021
5	7	9	5	6	5	6.93
0.54	0.61	0.62	0.63	0.69	0.69	
0.44	0.48	0.54	0.75	0.75	0.56	
0.202	**0.217**♦	0.134	-0.205	-0.086	0.202	0.029

*Tth*16-2								
N_A	7	8	6	5	6	4	6	7
H_E	0.60	0.62	0.61	0.63	0.65	0.61	0.65	0.72
H_O	0.50	0.50	0.58	0.61	0.60	0.64	0.61	0.54
F_{IS}	**0.162**	**0.194*◆**	0.051	0.043	**0.072**	-0.046	**0.056***	**0.254*◆**
Mean N_A	13.6667	13.9167	13.8333	14.75	15.666667	15.75	16.083333	16.916667
Mean H_E	0.80	0.81	0.80	0.81	0.83	0.81	0.83	0.83
Mean H_O	0.74	0.77	0.80	0.80	0.81	0.80	0.80	0.79
Multilocus F_{IS} p-value	**0.076***	**0.046***	0.000	0.008	**0.024***	0.016	**0.036***	**0.049***

5	6	5	3	4	4	5.43
0.60	0.59	0.60	0.57	0.48	0.61	
0.44	0.50	0.46	0.50	0.50	0.44	
0.278	0.160	0.230	0.118	-0.035	0.279	0.130
11.25	15.833333	16.666667	10.75	11.75	12.0833	
0.81	0.81	0.82	0.79	0.80	0.83	
0.78	0.77	0.79	0.81	0.81	0.82	
0.040	**0.053**	**0.038**	-0.030	**0.034**	-0.020	

Table 5.3. Frequency of null alleles using the Oosterhout Null Allele estimator as implemented in MICROCHECKER (Van Oosterhout *et. al.*, 2004) for sample and locus; - indicates no evidence for null alleles.

Locus	Med05	Med06	Med07	NEAtl05_1	NEAtl05_2	NEAtl05_3	NEAtl06_1	NEAtl06_2	NEAtl06_3	NEAtl07_1	NEAtl07_2	NEAtl07_3	Pac03	Pac05
*Ttho*6	-	-	-	-	-	-	0.026	-	-	-	0.060	-	-	-
*Tth*14	0.077	-	-	-	-	-	0.070	-	-	-	-	-	-	-
*Tth*254	0.019	0.010	-	-	0.010	-	-	0.007	-	-	-	-	-	-
*Tth*1-31	0.058	-	-	-	-	-	-	-	-	-	-	-	-	-
*Tth*12-29	-	-	-	-	-	-	-	-	-	0.087	-	-	-	-
*Tth*16-2	-	0.070	-	-	-	-	-	0.104	-	-	-	-	-	-

Table 5.4. Probability values for Fisher's exact tests of genic differentiation (below the diagonal) and pairwise F_{ST} values (above the diagonal; Weir & Cockerham, 1984) using corrected data for null alleles; P<0.05 in bold (P<0.0098 values with asterisk for 91 pairwise comparisons with B-Y method).

Sample	Med05	Med06	Med07	NEAtl05_1	NEAtl05_2	NEAtl05_3	NEAtl06_1	NEAtl06_2	NEAtl06_3	NEAtl07_1	NEAtl07_2	NEAtl07_3	Pac03	Pac05
Med05		**0.013***	**0.008***	**0.017***	**0.022***	**0.019***	**0.046***	**0.019***	**0.025***	**0.017***	**0.022***	**0.030***	**0.024***	**0.031***
Med06	**0.000***		0.002	**0.014***	**0.024***	**0.016***	**0.047***	**0.018***	**0.018***	**0.012***	**0.021***	**0.030***	**0.026***	**0.034***
Med07	**0.000***	**0.000***		**0.014***	**0.026***	**0.017***	**0.047***	**0.019***	**0.021***	**0.013***	**0.020***	**0.030***	**0.026***	**0.036***
NEAtl05_1	**0.000***	**0.000***	**0.000***		0.004	-0.001	**0.033**	0.001	-0.001	0.000	-0.001	0.003	**0.015***	**0.026***
NEAtl05_2	**0.000***	**0.000***	**0.000***	**0.001***		**0.005***	**0.033***	**0.003**	0.006	**0.005**	0.001	0.002	**0.006**	**0.015***
NEAtl05_3	**0.000***	**0.000***	**0.000***	0.485	**0.000***		**0.031***	**0.003**	0.001	0.002	0.002	**0.008**	**0.017***	**0.022***
NEAtl06_1	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***		**0.035***	**0.029***	**0.035***	**0.030***	**0.037***	**0.038***	**0.047***
NEAtl06_2	**0.000***	**0.000***	**0.000***	**0.001***	**0.000***	**0.000***	**0.000***		0.001	**0.003**	**0.003**	**0.006**	**0.015***	**0.020***
NEAtl06_3	**0.000***	**0.000***	**0.000***	0.501	**0.000***	0.160	**0.000***	0.216		0.006	-0.020	0.002	**0.010**	**0.018***
NEAtl07_1	**0.000***	**0.000***	**0.000***	0.346	**0.003***	**0.028**	**0.000***	**0.000***	**0.019**		0.001	0.006	**0.013***	**0.020***
NEAtl07_2	**0.000***	**0.000***	**0.000***	0.532	0.114	**0.047**	**0.000***	**0.000***	0.331	0.426		0.002	**0.009**	**0.019***
NEAtl07_3	**0.000***	**0.000***	**0.000***	0.087	0.195	**0.017**	**0.000***	**0.005***	0.213	0.146	0.458		**0.015***	**0.028***
Pac03	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***		-0.004
Pac05	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	0.601	

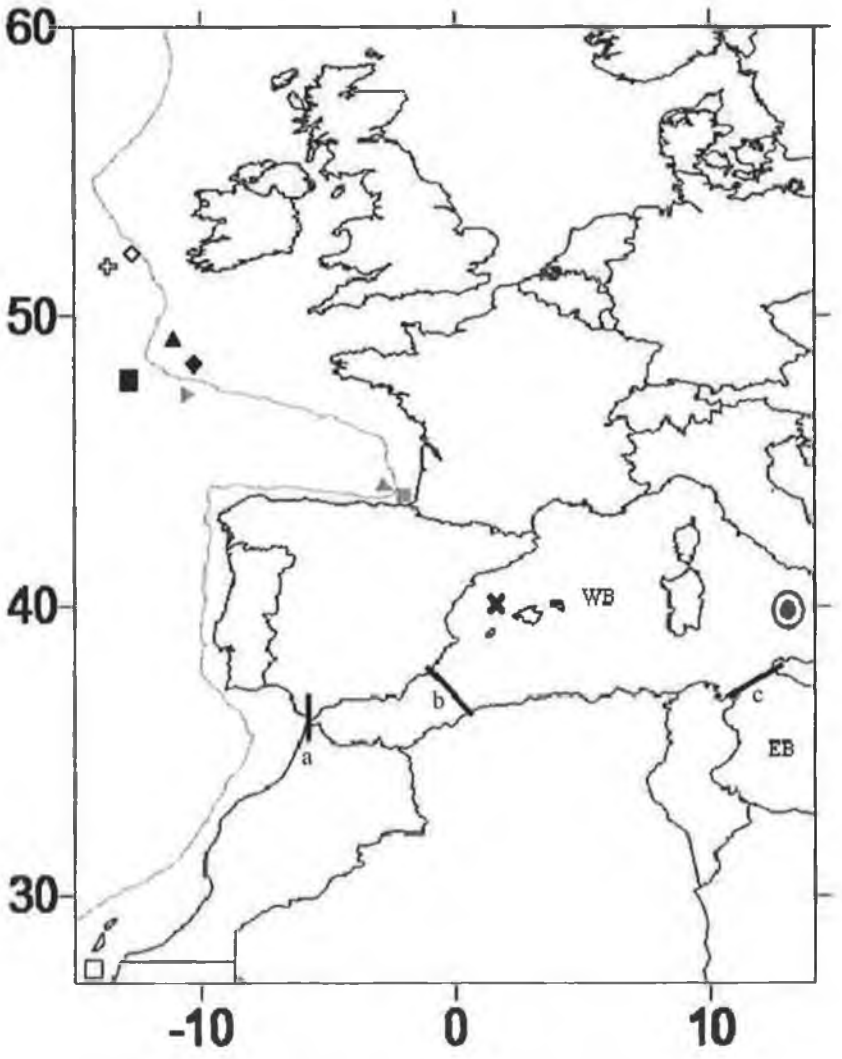

Figure 5.1. Geographical locations of albacore sampling in Med05 (X), Med06 (●), Med07 (○), NEAtl05_1 (▲), NEAtl05_2 (■), NEAtl05_3 (♦), NEAtl06_1 (►), NEAtl06_2 (▲), NEAtl06_3 (■), NEAtl07_1 (◊), NEAtl07_2 (+) and NEAtl07_3 (□). Solid black lines indicate a) the Straits of Gibraltar, b) the Almeria-Oran front and c) the Siculo-Tunisian front; grey line indicates approximate location of the Porcupine ridge (bathymetry 200m); the Goban spur is near ■; WB indicates Western Mediterranean Basin, EB indicates Eastern Mediterranean Basin.

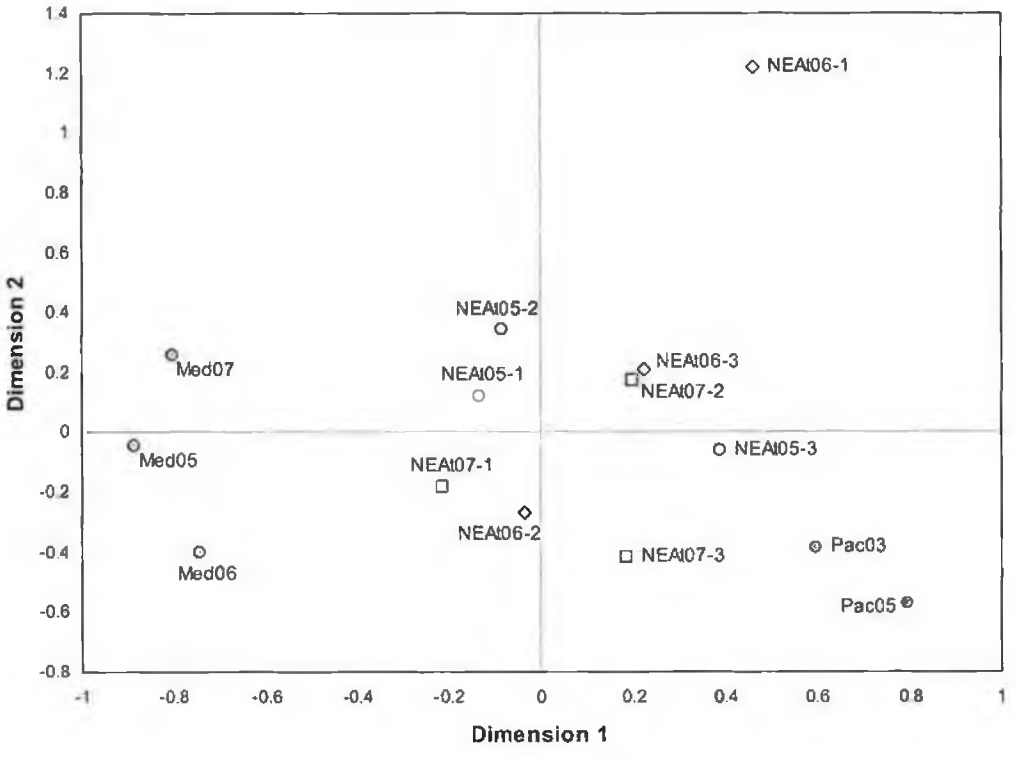

Figure 5.2. Multi dimensional scaling (MDS) plot of the relationship in space between one sample and another based on pairwise F_{ST} values.

Chapter 6: A holistic stock analysis of albacore tuna from the North Atlantic Ocean and Mediterranean Sea

6.1 Abstract

Stock discrimination and identification of populations contributing to mixtures are an important component of fisheries management. A wide variety of techniques have been used singularly to achieve definitive population separation in many different fish species. For some species a single method may be sufficient to categorise stocks, however a range of parameters used in conjunction can more accurately illustrate the stock composition of a fishery. In this study the ability of a variety of stock discrimination techniques to differentiate between samples taken from two separate stocks of albacore (*Thunnus alalunga*), the North Atlantic and Mediterranean, was tested. Differences within each stock were tested across two separate sampling years, 2005 and 2006. Differences were observed in the length-weight relationship, with North Atlantic fish as the greater of the two. ANOVA indicated differences between regions in four otolith chemistry variables and five shape variables. Discriminate function analysis based on otolith chemistry produced a classification success of 60% and shape variables classified 42% of fish correctly. A forward stepwise DFA combining otolith chemistry ($CoreMg^{26}$ (log_{10}) $CoreSr^{86}$ and $EdgeSr^{86}$ (inverse)) and shape variable (C23) classified 63% of fish accurately to area and year of origin. Five microsatellite loci were shown to contribute towards 85.1% successful classification of individuals to sample of origin. Of these techniques, differentiation of albacore in the North Atlantic and Mediterranean was highest using genetic methods.

Keywords: - Albacore, *Thunnus alalunga*, comparative methods, stock discrimination, age, growth, otolith chemistry, otolith shape, microsatellite DNA.

6.2 Introduction

Effective fisheries management is essential in order to obtain a balance between the harvest and conservation of stocks to prevent any future decline in abundance and productivity (Begg *et al.*, 1999a). The term "stock" can be defined in a number of ways. Waldman (1999) proposed the definition of a stock as a fisheries management unit, consistent with the concept of a population with some degree of genetic integrity. Whilst Begg *et al.* (1999a) defines the stock as "a semi-discrete group of fish with some definable attributes of interest to managers". The definition of a stock is dependant on the purpose for which the data is intended; some groups of fish may not be genetically distinct but may show differences in phenotypic characteristics and have low enough levels of exchange to warrant separate management. The aggregations targeted by commercial fisheries may comprise of single population units (either open or closed) or may be made up of several source populations, i.e. mixed stocks, which can contribute different proportions to the whole harvest. For example, Pacific salmon are often caught in oceanic or coastal areas where a mixture of several populations, in different proportions, forms the basis of the catch (Shaklee *et al.*, 1999). Identifying stocks, discriminating among them and determining the population composition of mixed stocks are integral elements of fishery management (Waldman, 1999).

There is no universal method for determining stock relationships in marine fishes (Smith *et al.*, 2002). Stocks were traditionally identified through observations of life history traits, such as large intra-annual differences in recruitment, timing of spawning activities, differences in growth rates or differences in morphology (Begg *et al.*, 1999b; Waldman, 1999). However more modern techniques are now being incorporated into stock discrimination studies, such as otolith microstructure (Brophy and Danilowicz, 2002; Berg *et al.*, 2005), microchemistry (Thresher, 1999; Rooker *et al.*, 2003), as well as genetic analysis (O'Connell and Wright, 1997; Was *et al.*, 2008). A wide variety of techniques have been used singularly to achieve definitive population separation in many different fish species. For some species a single method may be sufficient to categorise stocks, however a range of parameters used in conjunction can more accurately illustrate the stock composition of a fishery and its fluctuations (Smith *et al.*, 2002; Berg *et al.*, 2005; Miller *et al.*, 2005).

This study addresses stock discrimination in albacore tuna *Thunnus alalunga*, (Bonnaterre, 1788). Albacore is a highly migratory species of tuna, with a pan-oceanic distribution, inhabiting both tropical and temperate waters. In the Atlantic Ocean the northern and southern stocks are separated at 5°N, with a separate management unit in the Mediterranean Sea (ICCAT, 1996). The North Atlantic and Mediterranean populations are considered separate stocks due to differences in growth (Megalofonou, 2000), spawning areas and migration (Beardsley, 1969; Ueyanagi, 1971; Piccinetti and Piccinetti Manfrin, 1993) coupled with a lack of evidence of movement through the Straits of Gibraltar (Arrizabalaga *et al.*, 2003). However at this time the life cycle of North Atlantic and Mediterranean albacore tuna is still poorly known (Santiago and Arrizabalaga, 2005).

Otolith microchemistry investigations have previously been effective in discerning spatial structuring in some species of fish (Campana and Thorrold, 2001). The chemical composition of otoliths can be attributed to a wide variety of factors including, surrounding water chemistry (Farrell and Campana, 1996; Gillanders and Kingsford, 1996; Bath *et al.*, 2000; Milton and Chenery, 2001; Lin *et al.*, 2007), temperature (Kalish, 1989; Radtke *et al.*, 1990), and food sources (Gallahar and Kingsford, 1996). Otolith microchemistry studies investigating population structure typically employ two approaches (Thresher, 1999). Firstly, otolith chemistry analysis of larval or juvenile otoliths is used to create a micro-chemical profile of natal origin which can be compared to the chemical signature of the natal region in otoliths from older fish collected at a later date (Thorrold *et al.*, 2001; Patterson and Kingsford, 2005). Secondly, individuals are collected from across a broad region and geographic groupings are sought based on otolith elemental signatures (Thresher, 1999; Campana and Thorrold, 2001). Studies have described stock configurations (Campana *et al.*, 1994; Gillanders, 2001), site fidelity (Thorrold *et al.*, 1997), sample origin (Secor and Zdanowicz, 1998; Rooker *et al.*, 2001a; Brophy *et al.*, 2003; Rooker *et al.*, 2003) and migration patterns (Gemperline *et al.*, 2002; Stransky *et al.*, 2005) in different species of fish using these methods. Previous micro chemical investigations have provided valuable insights into the stock structure of other tuna species (Radtke and Shepherd, 1991; Proctor *et al.*, 1995; Rooker *et al.*, 2001a; Rooker *et al.*, 2003).

Morphometric parameters describing the relative size of various body parts and otolith shape can also be used as stock delineators (Cadrin and Friedland, 1999). Differences in the anatomy of different species of fish can relate to specific life

history patterns such as feeding strategy or mode of swimming (Wootton, 1999). Anatomical differences are also present in fish of the same species inhabiting different environments allowing for stock differentiation (Royce, 1964; Balbontin *et al.*, 1973; Von Cramon-Taubadel *et al.*, 2005; Bhagat *et al.*, 2006; Marcil *et al.*, 2006). Otolith shape variation can be detected using qualitative visual assessment (Berg *et al.*, 2005) or quantitatively using Fourier or Euclidean shape analysis (Castonguay *et al.*, 1991; Friedland and Reddin, 1994; DeVries *et al.*, 2002; Stransky and MacLellan, 2005; Petursdottir *et al.*, 2006; Burke *et al.*, 2008). There are no published works on the potential use of otolith shape as a stock discriminator in *Thunnus* species. However, Fourier analysis of otolith shape has proved useful in stock discrimination in other scombrid species which show similar otolith morphology (Castonguay *et al.*, 1991; DeVries *et al.*, 2002; Stransky *et al.*, 2008).

Genetic variation between populations or genetic stocks can be ascertained by a variety of molecular methods including protein and allozymes analysis, mitochondrial DNA markers and nuclear DNA markers (Ferguson, 1994). Although genetic distinction can be used as to differentiate between stocks it is not always a fail-safe method as some populations may not show genetic distinction but may be discrete for the purposes of management (DeVries *et al.*, 2002). Phenotypic markers may be used to detect stock structure in the absence of genetic heterogeneity (Brophy *et al.*, 2003; Burke *et al.*, 2008). Non-genetic markers which reflect differences in environmental conditions may provide additional insight into the origins and life-history parameters of genetically distinct groups, such as in albacore where stock heterogeneity has been shown between the North Atlantic and Mediterranean Seas (Chapter 5).

The use of multiple techniques has been referred to as the holistic (Begg and Waldman, 1999) or comparative (Waldman, 1999) approach to stock analysis. Some studies corroborate evidence of stock structure from one technique by comparing with results from other stock discriminators (Berg *et al.*, 2005; Miller *et al.*, 2005; Bradbury *et al.*, 2008). Although these studies can indicate complementary findings using different analysis types, in some cases, results can vary according to the technique used (Smith *et al.*, 2002), highlighting that stock structure may not be fully described by studies that focus on one particular method.

In this study, we used data describing age and growth, otolith microchemistry, and otolith shape to further examine population structure of albacore tuna in the North

Atlantic Ocean and Mediterranean Sea which has been inferred from microsatellite DNA analysis (Chapter 5). The primary questions were: (i) Are there differences in growth parameters between fish from the two populations? (ii) Is there any evidence that fish from genetically distinct samples from within each region were exposed to different environments as larvae? Or have these fish occupied different environments in the recent past? (iii) How does the discriminatory power of phenotypic markers compare to that provided by genetic data?

6.3 Materials & Methods

Sample Collection

Albacore were obtained onboard commercial vessels by Bord Iascaigh Mhara (BIM) to the southwest of Ireland along the Porcupine ridge in the North Atlantic Ocean and by Instituto Tecnológico Pesquero y Alimentario (ATZI), Spain from fish merchants in Spain (Balearic Sea) in 2005 and Southern Italy (Tyrrhenian Sea) in 2006 (Figure 6.1). Fish were caught by trolling in the Atlantic and Balearic Sea, and by pelagic long-lining in the Tyrrhenian Sea. A minimum of fifty fish were obtained from each area. Length and weight measurements were taken and are summarised in Table 6.1. The first dorsal fin ray spine was removed for ageing purposes. The heads were severed and frozen for up to 3 months before removal of the otoliths in the laboratory. Once extracted, the macula tissue on the otolith was removed using forceps and the otoliths were soaked in ultra pure water to remove any adhering tissue or fluids. They were stored in sterile acid washed plastic tubes for transport. Two samples were available for each stock for the otolith chemistry, shape and fish condition analysis, these sample have been shown to be genetically distinct (Chapter 5: NEAtl05_2; NEAtl06_1_1; Med05 andMed06). The same samples were used in age assessment and growth analysis, however this data was combined for each stock (NEAtl = NEAtl05_2 + NEAtl06_1; Med = Med05 + Med06).

Age and growth

The first spine from the first dorsal fin ray was imbedded in epoxy resin. Three sections of 1mm thickness were made following protocols presented by Oritz de Zárate *et al.* (2007a). The sections were placed on a slide and photographs of each section were taken at 1.6x magnification. Mean ages for each sample are shown in Table 6.1. Age frequency distributions are presented in Figure 6.2. Growth of albacore was estimated for the (von Bertalanffy, 1938) growth curve:

$$L_t = L_\infty \left\{ 1 - e^{\left[K^{(y^{-1})} (t - t_0^{(y)}) \right]} \right\}$$

Where L_∞ is the length at maximum age, K = growth per year and t_0 is the theoretical length at age 0. Reported growth rates for albacore vary between years and

geographic areas (Santiago and Arrizabalaga, 2005). The growth parameters proposed by ICCAT (2007) are shown in Table 6.2 with parameters calculated for North Atlantic and Mediterranean samples combined from 2005 to 2006 using Microsoft Excel Solver routine. The growth parameters were compared using log-likelihood comparison (Kimura, 1980). This method determines if the two datasets are best described by a single growth curve or by two region-specific growth curves, with unique values for some or all of the growth parameters. The data was tested for simultaneous equality of the three growth parameters. If significant differences were detected, further tests were carried out to evaluate differences between curves for each parameter in turn. Estimated von Bertalanffy growth parameters for the North Atlantic and Mediterranean samples were compared to the ICCAT parameters by reducing both data sets to the equivalent average size at age data.

The relationship between whole mass and fork length follows a power curve expressed by the equation: $y=cx^b$, where y = weight (kg), x = fork length (L_F), b and c are constants. Length and weight data for the North Atlantic and Mediterranean fish were natural-log (ln) transformed and the slope and intercept of the linear relationship calculated using least squares regression. Fulton's condition factor (C) was computed for each fish:

$$C = \frac{W}{L^3}$$

Where W is the weight of fish and L is the fork length (Stevenson and Woods Jr, 2006). Residual condition was calculated as the residual for each fish from the regression of ln weight versus ln length. Differences in the Fulton's condition and residual condition factor between the two regions were compared by univariate ANOVA.

Otolith Microchemistry Analysis

Otolith microchemistry was analysed using a New Wave Research (California) frequency quintupled Nd:YAG laser (λ = 213nm) coupled with a VG Elemental Plasma Quad 3 Inductively Coupled Plasma Mass Spectrophotometer (Thermo Elemental, Cheshire). A laser spot size of 40μm, a laser firing rate of 10Hz and a fluence of 0.3 J/cm^2 were set for analysis. Background levels of strontium and

calcium were monitored for elevated levels. No data were collected during periods with elevated background levels.

The percentage of CaO in otoliths was ascertained on a Varian Vista-Pro ICP-AES which had been calibrated with synthetic standards. An average value of 35% total calcium was present in otoliths. The minor isotope of calcium, ^{43}Ca, was used as the internal standard for data collection. The elements B^{11}, Mg^{24}, Mg^{26}, Mn^{55}, Zn^{66}, Sr^{86}, Sr^{88} and Ba^{137} were chosen for investigation. Data were processed using Laser Ablation specific macros within the package, Lotus 1-2-3.

Transverse sections of an otolith imbedded in epoxy resin were polished on silicon carbide wet-dry polishing paper to expose the core. During preparation the otoliths were cleaned of adhering material or fluids using ultra pure water. Samples were mounted on a slide (47mm x 25mm) in a Perspex ablation cell, which was placed on a motorized stage under a transmitted light microscope. The standard reference material (SRM) used was glass reference NIST 612 from the National Institute of Standards and Technology (Gaithersberg, Maryland, USA). Samples were analysed in sets of 20 ablations, the SRM was analysed twice at the start of a run and twice at the end to account for any instrumental drift. Ablation points were taken in the natal region (Core) and at approximately 80μm from the edge (Edge), which was deposited during the last 3 months of the fish's life.

Data were tested for outliers against Grubb's critical values and any outliers found were replaced with the next highest or lowest data point. One data point in each of $EdgeMg^{24}$, $EdgeMg^{26}$ and $EdgeBa^{137}$ and three data points in $EdgeSr^{88}$ were standardized against Grubb's critical values. Data were tested for equal variances across groups and for normality within groups, using Levene's statistic and Anderson-Darling test, respectively in Minitab® v.15 for Windows. A significance level of $\alpha \leq 0.05$ was used in all tests. Data were transformed, using the inverse or $\log_{10}$ functions as appropriate, to conform to homogeneity of variance and normality. Any variable (i.e. element) that did not conform was removed from further analysis. Univariate ANOVA's with *a posteriori* Tukey's tests were conducted to compare concentrations of each element between samples. Differences were observed in all elements except Mn^{55}, therefore this element was excluded from further analysis. The variables which showed significant variation between samples and were thus considered potentially useful for discriminate function analysis (DFA) were as

follows: CoreB^{11}, CoreMg^{24} ($\log_{10}$), CoreMg^{26} ($\log_{10}$), CoreSr^{86}, CoreSr^{88} ($\log_{10}$), EdgeMg^{26}, EdgeSr^{86} (inverse), and EdgeBa^{137}. To ensure that DFA would not be affected by multi-colinearity, variables were tested for univariate correlation within each group using Pearson's correlation coefficients in SYSTAT v.11 for windows. Multi-colinearity can result in the use of redundant predictors and can affect the outcome of the discriminate function analysis (Graham, 2003). Variables that were strongly correlated (correlation coefficient >0.5, $P<0.05$) were excluded from further analysis. The elements indicating greatest differences between samples but with no within or across group correlations were CoreMg^{26} ($\log_{10}$), CoreSr^{86}, EdgeMg^{26} and EdgeSr^{86} (inverse). These were included in the subsequent discriminate function analysis.

Otolith Shape Analysis

Images of the internal face (Figure 6.2) of both sagittal otoliths, where available were digitized using an Olympus™ Camedia digital camera and an Olympus™ SZX7 stereomicroscope at 12.5x magnification. The otoliths were photographed as a light object against a black background for maximum contrast. During otolith extraction many otoliths were broken at the rostrum end. DeVries *et al.* (2002) showed that partial images of king mackerel otoliths could be used in stock separation; therefore crops were made of broken otolith images at easily recognisable features i.e. through the core. The image analysis was conducted using Image Pro Analyzer v.6.2. The outline of each otolith was traced digitally and the size parameters (area, perimeter, feret length and feret width) were measured. Feret length and feret width are the length and width of a box, respectively, which encloses the otolith outline. The size parameter measurements were used to calculate a number of shape indices outlined in Table 6.2. The images were converted to monochrome bitmaps and saved for further analysis.

Using the bitmap images digitised outlines were generated using TpsDig[4] image analysis software. The outlines were saved as a series of x, y co-ordinates as TPS files. The user can accept the number of co-ordinates generated by TpsDig or can select a lower number of co-ordinates. The actual numbers of co-ordinates

[4] Public domain program developed by F.J. Rholf, available freely on the internet at http://life.bio.sunysb.edu/morph/

generated were used in this investigation. TPS files were converted into a single data file in the format required for the Fourier analysis program EFAwin[5].

In EFAwin, 20 shape harmonics were generated for each otolith. Each harmonic consists of four coefficients resulting in eighty coefficients (C) per individual. The program standardises for size and orientation, the first three coefficients are therefore fixed values of C1=1, C2=C3=0. Each individual is therefore represented by 77 unique coefficients (C4-C80). Further details on Elliptic Fourier Analysis can be found in Lestrel (1997).

Shape indices and coefficients (henceforth referred to collectively as shape variables) were examined for normality and homogeneity of variance using MINITAB® 15. Variables that conformed to these assumptions were tested for between sample for differences using univariate ANOVA, with Tukey's *post hoc* test to ascertain the direction of any observed differences. Based on the results of the ANOVAs, a suite of shape variables were selected from the original shape indices and shape coefficients. Four shape indices and 10 shape coefficients showed significant variation between samples and so were considered potentially useful for discrimination. Variables were tested for univariate correlations within each group using Pearson's correlation coefficients in SYSTAT 11 for windows. Any variables that were strongly correlated (correlation coefficient >0.5, $P<0.05$) were removed from further analysis. Variables were also tested for significant correlation (>0.5, $P<0.05$) with otolith length. If a significant correlation was detected, ANCOVA was carried out with sample included as a fixed factor and otolith length (of the cropped image) included as a covariate to determine if the relationship between otolith length and the shape variable was consistent between samples (interaction term: $P<0.05$). A significant interaction was observed for C64 and this variable was excluded from further analysis. Where significant correlation existed and ANCOVA revealed no sample*otolith length interaction, the size effect was corrected using the common within group slope (b) (Turan, 2000; DeVries *et al.*, 2002; Tuset *et al.*, 2006). This procedure was used to adjust circularity (Table 6.3). The adjusted variable showed no significant correlation with otolith length ($P>0.05$).

[5] Public domain program developed by F.J. Rholf and S. Ferson, available freely on the internet at http://life.bio.sunysb.edu/morph/

Discriminate Function Analysis using Otolith Chemistry and Shape Variables

Variables were tested for homogeneity of the covariance matrices by carrying out Box's M test using PAST version 1.89 (Hammer *et al.*, 2001) to ensure the variables meet the assumptions of MANOVA (Gotelli and Ellison, 2004). The test showed that this assumption was not violated ($P>0.05$) and therefore forward stepwise linear discriminate function was utilized (Seber, 2004). Three separate stepwise linear discriminate function analyses (DFA) were performed in SYSTAT version 11. The first included chemistry variables, the second shape variables and the third a combination of chemistry and shape variables. In each case the suite of variables included was based on the results of the univariate ANOVA's as outlined above. The forward stepwise model used an F to remove statistic of 4.0 to ascertain the individual variables with the greatest discriminatory power. The DFA procedure initially classifies each case into the group where the value of its classification function is largest. These results can be misleading as the classification rule is evaluated using the same cases that are used to compute it. The jack-knifed classification procedure, which was used in this analysis, attempts to remedy this problem by removing and replacing each case one at a time and using functions computed from all the data except the case being classified (Engleman, 2004).

Microsatellite DNA classification

Microsatellite DNA methodology using 12 microsatellite loci is shown in Chapter 5. The data for microsatellites unaffected by null alleles (Van Oosterhout *et al.*, 2004) were used for classification purposes. The program WHICHLOCI 1.0 was used to predict sample of origin based on microsatellite data (Banks *et al.*, 2003). Genotype data was re-sampled within the program and a user-defined sample size of 1000 was applied to test classification success for the four samples. Loci were ranked in order of contribution to the overall classification success of the test. Classification success using microsatellite DNA was compared to the classification success using otolith microchemistry and shape information.

6.4 Results

A total of 240 albacore were used in this study from four different geographic areas in the north east Atlantic Ocean and Mediterranean Sea (Figure 6.1). The mean fork lengths (L_F) of the fish used in the study ranged from 59.5 cm to 64.5 cm (Table 6.1). Age estimates indicated that the samples were dominated by two and three year old fish (Figure 6.3), however more three and four year old fish were observed in Mediterranean samples compared to those from the North Atlantic. The assigned ages ranged from 2-6 years.

Differences in growth parameters

Length at age was calculated using the Von Bertalanffy (1938) growth parameters. Estimated parameters for each sample are indicated in Table 6.4. Figure 6.4 shows the variation between the estimated growth curves between the two regions. Fish from the North Atlantic appeared to have a higher length at age compared to fish from the Mediterranean after age 3, this was confirmed by ANCOVA of fork length versus sample with age group as a covariate ($P<0.05$).

The log-likelihood test for equality of three Von Bertalanffy (1938) growth parameters revealed significant differences between samples for all pairwise comparisons (Table 6.5). Tests of individual parameters revealed significant differences in L_∞ and K between the North Atlantic and Mediterranean samples (Table 6.5). Significant differences were detected between all ICCAT parameters compared to the Mediterranean and North Atlantic, with the exception of t_0 in the North Atlantic. Individual comparisons indicate that the L_∞ estimate for fish collected from both areas is significantly lower than the estimate used by ICCAT in the assessment of the North Atlantic stock (Table 6.4).

Weight length relationships shown in Figure 6.5 indicate that albacore from the North East Atlantic have a greater weight to length ratio than Mediterranean albacore. The relationships for 2005 and 2006, North East Atlantic and Mediterranean were:

$$W = 3.483e^{-5}L_F^{2.8952} \text{ (NEAtl05\_2)}$$
$$W = 1.0974e^{-5}L_F^{3.1643} \text{ (NEAtl06\_1)}$$
$$W = 3.991e^{-5}L_F^{2.8138} \text{ (Med05)}$$

$$W = 1.568e^{-5}L_F^{3.0412} \text{ (Med06).}$$

The ICCAT standard length weight relationship as proposed in Santiago (1993) which follows the curve of: $W = 1.339e^{-5}L_F^{3.107}$ (for combined data for one fishing season from July to December, 1990). ANCOVA showed that the intercept of the length-weight relationship (using the natural-log transform to produce a linear relationship) was significantly higher for albacore from the North East Atlantic Ocean compared to fish from the Mediterranean Sea ($P<0.05$). There was no significant difference in the slopes of the relationships over the length range in the samples ($P>0.05$). Fulton's condition factor was higher in the North Atlantic samples at 2.26 (NEAtl05_2) and 2.15 (NEAtl06_1) compared to the Mediterranean samples 1.85 (Med05) and 1.86 (Med06). This was confirmed statistically by ANOVA (Table 6.6) where significant differences ($P<0.05$) were evident in between the two regions with the North Atlantic fish showing a greater Fulton's condition index than the Mediterranean. Post-hoc tests showed that the NEAtl05_2 sample had a higher mean condition than all the other samples ($P<0.05$) but there were no significant differences between NEAtl05_2, Med05 and Med06 ($P>0.05$). ANOVA of residuals again indicates that North Atlantic fish are larger than Mediterranean fish (Table 6.6; $P<0.05$) and this is also observed within samples as the NEAtl05_2 residual index is larger than all other samples ($P<0.05$) and NEAtl06_1 is larger than the Mediterranean samples (Med05 and Med06) ($P<0.05$). There are no significant differences between the Mediterranean samples (Med05 and Med06) in either Fulton's condition index or residual index ($P>0.05$).

Otolith elemental composition and otolith shape discriminate analysis

Chemistry data from the otolith core was available for a total of 144 fish. Edge chemistry data was available for 76 fish. A total of 211 otoliths were included in the shape analysis. The discriminate function analysis was restricted to the 76 fish for which core chemistry, edge chemistry and morphometric data were available. The sample sizes are as follows: Med05 (N=17), Med06 (N=19), NEAtl05_2 (N=18) and NEAtl06_1 (N=22).

The variables which showed the greatest between sample variations and were not correlated with each other were: $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeMg^{26}$ and $EdgeSr^{86}$ (inverse). Table 6.7 shows the results of univariate ANOVA of the four

chemistry variables. Tukey's post hoc test revealed otolith chemistry differences both within and between regions. Otolith core concentrations of Mg^{26} (log_{10}) were lower in the Med05 sample compared to the other samples; core concentrations of Sr^{86} were greater in Med06 otoliths compared to NEAtl05_2 and Med05 otoliths; otoliths from the NEAtl06_1 and Med06 samples had higher concentrations of Mg^{26} at the edge otolith edge compared to Med05; concentrations of Sr^{86} (inverse) at the otolith edge were greater in the NEAtl06_1 sample compared to the other samples.

Shape variables indicating within group differences in univariate ANOVA are shown in Table 6.7; these are circularity (adjusted), C8, C23 and C70. Tukey's post hoc test revealed otolith shape differences both within and between regions. Adjusted circularity was shown to be highest in NEAtl05_2, followed by NEAtl06_1 and then Med05 ($P<0.05$). For the shape variables C8 and C70, otoliths from the NEAtl06_1 sample had significantly higher mean values compared to otoliths from the Med05 variable. For the shape variable C23 mean values were higher for the Med06 otoliths compared to the NEAtl06_1 and Med05 otoliths.

When sample sizes are small, the inclusion of a large number of predictors in a DFA can artificially increase the classification success. As a general rule of thumb, the sample size in the smallest group should be at least three times greater than the number of predictors (Seber, 2004). In this analysis the smallest sample size was 17 (Med05), so the DFA should be restricted to five predictor variables. The discriminate function analyses were conducted by stepwise addition of predictor variables. Variables were included if the *F* to enter statistic was above the threshold 4.0 (which is automatically selected as threshold in SYSTAT v.11 stepwise DFA). The variable of $EdgeMg^{26}$ was omitted as too many measurements were below detection. Therefore the otolith chemistry DFA was based on $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$ and $EdgeSr^{86}$ (inverse). All three variables had F statistic above 4.0 and were therefore included in DFA. The discriminate function analysis returned a correct classification of 64% with a jack-knifed score of 60% for the three chemistry variables (Table 6.8 and 6.9), with a Wilks' Lambda (λ) value of 0.356, $P<0.05$. Canonical discriminate values (Table 6.10) indicate that the first discriminate function is mainly based on $EdgeSr^{86}$ (inverse) (0.747) followed by $CoreMg^{26}$ (log_{10}) (-0.672). The second function is primarily based on $CoreSr^{86}$ (0.995) followed by $EdgeSr^{86}$ (inverse) (0.649) then $CoreMg^{26}$ (log_{10}) (0.513). The third function is based on $CoreMg^{26}$ (log_{10}) (0.581), followed by $CoreSr^{86}$ (-0.469), and lastly $EdgeSr^{86}$ (inverse)

(0.421). Based on Eigen values function 1 contributes to 68% of classification, function 2 contributes 30% and function 3 the remaining 2%. The canonical scores of group means (Table 6.11) indicates that the first function distinguishes the Med05, from the others. The second function separates the Med06 sample from the NEAtl05_2 and NEAtl06_1 samples and the third function separates the NEAtl06_1 and NEAtl05_2 samples (Table 6.11). There is a high degree of overlap in the canonical score plot (Figure 6.6), although samples NEAtl06_1 and Med05 appear to be the most distinct.

The second DFA attempted to separate the four samples based on shape variables. The final model included two predictors: circularity (adjusted) and C23. The shape variables returned a poorer classification success of 42% and 42% jack-knifed (Table 6.12 and 6.13), and a Wilks' λ value of 0.693, $P<0.05$. The canonical discriminate variables show that function 1 is mostly based on C23 (0.656) and function 2 is mostly based on circularity (adjusted) (0.808) (Table 6.14). The Eigen values show that 58% of classification success is based upon the first function and the remaining 42% is based on function 2. The canonical score of group means (Table 6.15) shows that the first function maximises the separation of the NEAtl06_1 sample from the other three samples. The second function separates the Med05 from the Med06 sample and the NEAtl05_2 sample. A high degree of overlap between the four samples can be observed in the canonical score plot (Figure 6.7).

The third DFA combined all otolith chemistry and shape variables to distinguish between the four samples. The final model included four predictors: $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeSr^{86}$ (inverse) and C23. The DFA yielded a statistically significant result (Wilks' $\lambda = 0.283$, $F = 9.16$ and $P<0.05$). This DFA returned the highest overall classification success of 67% (Table 6.16), with a jack-knifed classification success of 63% (Table 6.17). Canonical discriminate values (Table 6.18) show that the first DFA function is based mainly on $EdgeSr^{86}$ (inverse) (0.839); followed by $CoreMg^{26}$ (log_{10}) (-0.557.), and the shape variable C23 (0.220). The second DFA function is primarily based on $CoreMg^{26}$ (log_{10}) (0.690), with $CoreSr^{86}$ as the next highest contributing variable (0.687) (Table 6.18). The third DFA function is based on $CoreSr^{86}$ (0.844) then the shape variable C23 (-0.738). Eigen values indicate that 57% of the variance between the samples can be attributed to the first function, and 36% and 7% to the second and third functions, respectively. The canonical scores of group means (Table 6.19) indicates that the first function

distinguishes the NEAtl06_1 sample from the other three. The second function separates the Med05 sample and the third function the NEAtl05_2 sample (Table 6.19). The canonical score plot (Figure 6.8) shows a high degree of overlap between the samples, but NEAtl06_1 appears to be the most distinct and is separated from the other groups by function 1.

Microsatellite DNA classification

Classification success was conducted in the program WHICHLOCI based on resampled populations (N = 1,000). The classification success was 85.1%. The samples were ranked in order of contribution to the classification success, five microsatellite loci out of a possible six contributed the most to the classification success, these are (in order); *Tth*4, *Tth*17, *Tth*185, *Tth*5 and *Ttho*7.

6.5 Discussion

Stock analysis using microsatellite DNA markers has shown that the four samples used in this study are genetically distinct; this is supported by both F_{ST} estimates and exact tests (Chapter 5). In light of these observed genetic differences this study assessed potential differences in phenotypic or environmental markers within albacore from the North East Atlantic and Mediterranean Sea from 2005 to 2006. Heterogeneity was observed in the length-weight and length at age relationships. Analysis of variance also indicated stock specific differences in a number of otolith shape parameters and in otolith elemental composition (Table 6.7). These differences may arise due to the underlying genetic variation or it may reflect differences in the environment experienced by the fish during the life history. As discussed below, it is likely that they at least partly reflect variation in the environmental conditions experienced by the fish.

Differences in Growth Parameters

The growth estimations in this study were based on small sample sizes (N<100) fished from the North East Atlantic Ocean and the central and western Mediterranean Sea over two sampling years. The age frequency histogram (Figure 6.2) shows that ages of fish ranged from two to six years, indicating that the majority of fish are immature juveniles, less than 5 years old (Santiago and Arrizabalaga, 2005). One and two year old fish were absent from the Mediterranean samples; this may be the result of size selectivity in the commercial fishery, previously unknown size-specific differences in movement patterns or high juvenile mortality within this area and sampling period.

The estimated Von Bertalanffy (1938) growth parameters (K, and L_∞) for the North Atlantic fish were significantly different to the ICCAT growth parameters derived by Bard, (1981) cited in ICCAT (2003). This may be due to the small sample sizes available in this study (N = 100). In addition, the age range of fish available in this study was restricted compared to Bard (1981) shown in Santiago and Arrizabalaga (2005). The weight length relationships observed in the Mediterranean fish were comparable to that described in Megalofonou (1990) for the Aegean Sea (1986 and 1989).

Information on the sex of the fish was not available for North Atlantic albacore but was for Mediterranean fish; therefore it was not possible to test for sexual differences in growth rates in the two stocks in this study. However sexually dimorphic growth rates have been observed by Megalofonou (2000) and Santiago and Arrizabalaga (2005), with males growing at a faster rate than females after the onset of maturation at around age 5. As 92% of the fish used in this study were considered as immature juveniles, sexual dimorphism may not have been evident.

Differences in the growth parameters were observed when growth curves for the Mediterranean fish were compared to growth curves for the North East Atlantic fish, and to the ICCAT growth curve. Fish from the North East Atlantic also had greater weights at length compared to fish from the Mediterranean (Figure 6.5). These results are consistent with previous reports of growth differences between these two areas (Megalofonou, 2000). Statistical analysis showed that Fulton's condition factor and residual index is higher in North Atlantic fish compared to Mediterranean fish, indicating the presence of fish in better condition i.e. fatter fish, in the North Atlantic. These differences could be attributed to a combination of environmental and genetic factors. Differences in growth rates of fish are often observed between different environments and along environmental gradients (Wootton, 1999; Vainikka *et al.*, 2009), and are related to the availability of food, temperature, salinity, etc. Growth rates and condition are also linked to physiological processes such as maturation (Wootton, 1999). However, some differences in growth may be an expression of genetic variability for ecologically relevant traits (Marcil *et al.*, 2006; Hutchings *et al.*, 2007; McDermid *et al.*, 2007). Regardless of their underlying cause, the observed differences in growth rates and condition between albacore from the North Atlantic and Mediterranean may have significant ramifications for the management of the Mediterranean stock. In spite of catch of approximately 6 tonnes per annum no stock assessments have ever been conducted for the Mediterranean stock (Anon, 2008). Therefore any future stock assessments would need to take into consideration the unique life history characteristics of this stock.

Differences in condition were also observed between the two North Atlantic samples. Fulton's condition index and residual indices indicated significant differences between NEAtl05_2 and NEAtl06_1, with NEAtl05_2 as the larger in both cases. This may indicate variation in the condition of genetically distinct albacores; this may warrant further investigation.

Otolith Chemistry

The factors affecting otolith composition are widely debated, with environment and selective metabolism as the main contributing factors (Campana, 1999), however it is likely that the expression of the genotype i.e. the phenotype, has a role in determining the physiology and metabolic parameters of individual fish. The influence of genetics on otolith composition and shape is poorly known, however it has been indicated that the level of successful classification of fish to a particular stock using otolith shape parameters increases with genetic discreteness or geographic separation (Castonguay *et al.*, 1991; Friedland and Reddin, 1994). This suggests that otolith shape variation is determined to some degree at least by genotype. Contrary to this, many fish stocks that are genetically similar do show variation in growth rates (Brophy and Danilowicz, 2002), otolith chemistry (Begg, 1998; Thorrold *et al.*, 2001; Brophy *et al.*, 2003; Geffen *et al.*, 2003) and shape (Leslie and Grant (1990); Pippin and Carr (1993) both as cited in Begg and Waldman (1999); and Burke *et al.* (2008)).

Differences in otolith chemistry have been attributed to a wide range of environmental factors including water chemistry, salinity, temperature, and food availability. Although this study does not investigate the influencing factors of otolith chemistry, it is known that differences exist in the oceanographic features between the two water bodies, and differences in diet of Mediterranean and North Atlantic albacore have been presented (Pusineri *et al.*, 2005; Goñi and Arrizabalaga, 2007). The analysis of variance indicated that significant differences were present in the concentrations of magnesium and strontium between the North Atlantic and Mediterranean samples in both the core and edge of the otoliths.

No differences were observed in core chemistry between the two North Atlantic samples. Genetic distinction between the two North Atlantic samples suggest the possibility of distinct larval origins, however the environmental differences in core chemistry show insufficient variation to be able to distinguish between these two samples. The two Mediterranean samples (Med05 and Med06), which have been shown to be genetically distinct, showed small variation in otolith core strontium. This may occur because the fish were exposed to different ambient levels of strontium, to different feeding conditions or to different temperature regimes during larval development. Previous studies have shown that strontium calcium ratios are

influenced by water temperature (Radtke *et al.*, 1990; Sadovy and Severin, 1992; Townsend *et al.*, 1992; Townsend *et al.*, 1995; Friedland *et al.*, 1998), environmental availability (Farrell and Campana, 1996) and growth patterns (Sadovy and Severin, 1994; Clarke and Friedland, 2004). It is possible that Mediterranean fish may originate from more than one spawning ground, as both adult and larval albacore distribution has been shown to be discontinuous (Piccinetti and Piccinetti Manfrin, 1993; Piccinetti *et al.*, 1997; Megalofonou, 2000). The effects of inter-annual variation cannot be ruled out completely without further investigation.

Although there is strong evidence that the fish in the North Atlantic and Mediterranean have distinct larval origin (Chapter 5 of this study; Ueyanagi, 1971; Piccinetti and Piccinetti Manfrin, 1993; Piccinetti *et al.*, 1997; Arrizabalaga *et al.*, 2004; Viñas *et al.*, 2004; Nakadate *et al.*, 2005), there is not evidence of a strong elemental fingerprint in the otolith core. The application of otolith chemistry to reconstruct the environmental history of pelagic spawning fish has been limited (Ashford *et al.*, 2005). Some studies have shown good levels of classification success of pelagic fish to spawning grounds (Rooker *et al.*, 2003; Ashford *et al.*, 2005), whilst others have met with more modest success (Stransky *et al.*, 2005). Although it is possible to characterize some oceanic species of pelagic spawning fish to natal origin, as the gradients in physical and chemical properties are less extreme in the open ocean. Otolith chemistry may be more useful in characterizing fish which present inshore spawning, or have nursery areas in estuaries or close to reefs, where a unique and stable geochemical signature is more likely to arise. It can be concluded from this study that otolith microchemistry from LA ICPMS may not be a useful method for characterising spawning areas in albacore, however further work using alternative microchemical techniques, such as stable isotope analysis, should not be discounted and may be able to provide further insight into albacore origin and migrations.

The chemistry of the otolith at the edge corresponded to approximately 3 months prior to capture (determined by mean microincrement width). Significant differences were observed in strontium in the two Atlantic samples at this point in the fish's life. This suggests that these fish were exposed to different environmental conditions prior to or during the migration to the summer/autumn feeding grounds in the North East Atlantic. The differences may indicate that the fish follow distinct migration pathways to the feeding areas (Hue, 1980) or it may be attributed to inter-annual differences in the environment experienced by fish following the same

migration pathway. A more intensive sampling program within a single year would be required to ratify or refute these arguments. Differences were observed in magnesium at the edge of Mediterranean albacore otoliths. These again possibly reflect differences in environmental conditions experienced by the fish before capture.

Otolith Shape

Previous studies on Fourier series otolith shapes in scombrids have proved useful in identifying individuals in mixed stocks of king mackerel (*Scomberomorus cavalla*) (DeVries *et al.*, 2002) and horse mackerel (*Trachurus trachurus*) (Stransky *et al.*, 2008). In this investigation, small levels of otolith shape variation were detected within and between Mediterranean and North Atlantic albacore. This provides further evidence to corroborate the hypothesis that the fish were exposed to different conditions during their life history. Otolith shape can be affected by a range of factors. Campana and Casselman (1993) showed that the shape of cod otoliths was dependant on stock specific growth rates and indicated differences among age, sexes and year classes. Cardinale *et al.* (2004) concluded that stock and environmental influences were significant in determining otolith shape. As growth is often dependant on food availability, species specific differences in diet may also contribute to differences in otolith shape (Gagliano and McCormick, 2004). The discriminate analysis in this study yielded poor results in classification success. This indicates that the level of shape variation is small and otolith shape variables are not strong enough to classify any potential differences between fish which have been separated by 1000's of km for their entire lives (North Atlantic and Mediterranean Sea). Therefore it can be concluded that otolith shape is a poor descriptor of stock structure in albacore.

The combination of otolith chemistry and shape variables as a comparative or holistic analysis yielded a greater rate of classification success than by one of the individual methods. Prior probabilities, based on random chance and sample size predicts that an average of 25% of fish would be correctly classified. The highest jack-knifed classification success achieved in this investigation was 63%. This is only marginally better than classification success using otolith chemistry alone, therefore it can be concluded that shape does not add significantly to the overall classification success. Many studies using otolith shape as a stock predictor have encountered

success rates ranging from 60 to >90% (Bird *et al.*, 1986; Castonguay *et al.*, 1991; Gauldie and Jones, 2000; Smith *et al.*, 2002; Cardinale *et al.*, 2004; Berg *et al.*, 2005). Whereas in otolith chemistry studies classification success ranged from <50% to >90% (Wells *et al.*, 2000; Stransky *et al.*, 2005; Veinott and Porter, 2005). The level of classification success in this study are comparable to success rates in other studies however the overall classification success is much lower than that observed by (DeVries *et al.*, 2002; Rooker *et al.*, 2003) in shape and chemistry studies in other scombrid species.

Microsatellite DNA Classification Success

The program WHICHLOCI has been used to successfully classify fish to origin populations (Tranah *et al.*, 2004; Miller *et al.*, 2005; Nikolic *et al.*, 2009). The classification success rates ranged from 74.6% using 7 loci in rockfish (Miller *et al.*, 2005) to 98% using 28 markers in salmon (Nikolic *et al.*, 2009) and 98% using 10 microsatellite loci in sturgeon (Tranah *et al.*, 2004). In this study 85.1% of fish were correctly classified to sample origin using 6 microsatellite loci. This is comparable to classification success in other studies.

Comparison of Classification Success by Otolith Chemistry and Shape versus Microsatellite DNA

It has been shown that very little exchange occurs between North Atlantic and Mediterranean albacores (Arrizabalaga *et al.*, 2002; Arrizabalaga *et al.*, 2003) and that there are significant genetic differences between all samples (Chapter 5) covering a geographic area from the southwest of Ireland through to the Tyrrhenian Sea in the Mediterranean (Figure 6.1). The highest classification success was provided by microsatellite DNA data at 85%. Otolith micro-chemistry may provide additional insight in situations where genetically similar stock components follow divergent migrations. Shape adds very little to the classification of the samples. It has been suggested that other morphometric features may yield differences between stocks; body shape variation, specifically head length to body length ratio (Ishii, 1965), is suspected to vary between tuna stocks (Royce, 1964; Schaefer, 1991; Penney *et al.*, 1998). Further investigations into albacore stock differentiation using multi

disciplinary methods should include collection of data on body shape variation, otolith microchemistry (from solution based ICPMS of whole otoliths or micro-milled portions of otoliths) and microsatellite DNA throughout the fishing season (July to November) across a wider geographic range in the North Atlantic Ocean and Mediterranean Sea to ascertain if a greater degree of stock separation can be achieved. However it appears at this time that microsatellite DNA data provides the most powerful discriminatory power for separating individual albacores between the North Atlantic and Mediterranean stocks.

Table 6.1. Mean length, weight and age, ± 95% C.I., of albacores caught in the North East Atlantic 2005 and 2006 (NEAtl05 and NEAtl06), Balearic Sea 2005 (Med05) and Tyrrhenian Sea 2006 (Med06).

	Number of fish in sample (N)	Mean Length (cm)	Mean Weight (Kg)	Mean Age
NEAtl05	71	62.0 ± 7.9	5.62 ± 2.07	2.86 ± 0.74
Med05	52	63.2 ± 2.6	4.68 ± 0.61	3.61 ± 0.57
NEAtl06	67	59.5 ± 8.5	5.00 ± 2.60	2.56 ± 0.86
Med06	50	64.5 ± 4.0	5.10 ± 1.00	3.85 ± 0.83

Table 6.2. Size parameters and resulting shape indices calculated for analysis of each sagittal otolith.

Size Parameters	Shape Indices
Area (A)	Circularity = P/A^2
Perimeter (P)	Rectangularity = A / (FL*FW)
Feret Weight (FW)	Form-Factor = $(4\pi A)/P^2$
Feret Length (FL)	Roundness = $(4A) / (\pi FL^2)$
	Ellipticity = (FL-FW) / (FL+FW)

Table 6.3. Pearson's correlation coefficients for shape variables against otolith length as a covariate as selected for discriminate function analysis; p values are indicated in parenthesis, b = within group slope of sample*otolith length interaction.

	Otolith Length	b
Circularity	-0.202 (>0.05)	-0.000023
Circularity (adjusted)	0.029 (>0.05)	
C8	0.090 (>0.05)	
C23	0.150 (>0.05)	
C70	-0.076 (>0.05)	

Table 6.4. Von Bertalanffy Growth Function parameters proposed by ICCAT (2007) and those for combined NE Atlantic 2005 to 2006 and Mediterranean 2005 to 2006 in this study. K = growth per year, L_∞ is the length at maximum age, and t_0 is the theoretical length at age 0.

Model	$K(y^{-1})$	L_{∞} (cm)	t_0 (y)
NEAtl (05 and 06 Combined)	0.37	89.09	-0.59
Med (05 and 06 Combined)	0.13	96.89	-4.39
ICCAT	0.23	124.74	-0.90

Table 6.5. Log-likelihood ratio (χ^2) of Von Bertalanffy individual growth parameters and coincidence of all growth function parameters estimated for NEAtl05, NEAtl06, Med05 and Med06 (comparisons versus ICCAT were reduced to average size at age data to coincide with available ICCAT information) (P<0.05 emboldened, P<0.01 with asterisks).

	Parameter	NEAtl (05 and 06 Combined)	Med (05 and 06 Combined)
Med (05 and 06 Combined)	Coincident	**324.62***	-
	L_∞	**-3.75***	-
	K	**-2.53***	-
	t_0	1.58	-
ICCAT	Coincident	**40.66***	**60.17***
	L_∞	**<0.01***	**27.28***
	K	**0.04**	**6.87***
	t_0	0.30	**17.26***

Table 6.6. Results of univariate ANOVAs comparing Fulton's Condition Index and Residual Index between samples.

	Mean-square	DF	F	P	r^2	Tukey's Post Hoc Tests
Fulton's C. I. NEAtl vs. Med	3.761	1	26.39	<0.001	10.38	NEAtl>Med
Fulton's C. I. of NEAtl05, NEAtl06, Med05 and Med06	2.431	3	18.97	<0.001	20.12	NEAtl05>NEAtl06; NEAtl05>Med05; NEAtl05>Med06
Residual Index NEAtl vs. Med	1.538	1	321.85	<0.001	59.18	NEAtl>Med
Residual Index of NEAtl05, NEAtl06, Med05 and Med06	0.544	3	123.68	<0.001	62.78	NEAtl05>NEAtl06; NEAtl05>Med05; NEAtl05>Med06; NEAtl06>Med05; NEAtl06>Med06

Table 6.7. Results of univariate ANOVAs comparing otolith chemistry and shape parameters between samples.

	Mean-square	DF	F	P	r^2	Tukey's post-hoc tests
CoreMg^{26} (log_{10})	0.392	3	10.77	<0.001	31.27	NEAtl06>Med05; Med05<Med06
CoreSr^{86}	389448	3	5.11	0.003	17.56	NEAtl05>NEAtl06;NEAtl06<Med05; NEAtl06<Med06
EdgeMg^{26}	124.48	3	5.94	0.001	22.89	NEAtl05>Med05; NEAtl06>Med05; Med05<Med06
EdgeSr^{86} (inverse)	<0.001	3	13.66	<0.001	36.28	NEAtl05<Med06; Med05<Med06
Circularity (adjusted)	0.003	3	4.97	0.003	17.15	NEAtl05>NEAtl06; NEAtl06>Med05
C8	0.001	3	3.39	0.023	12.36	NEAtl06>Med05
C23	<0.001	3	5.02	0.003	17.31	NEAtl06<Med06; Med05<Med06
C70	<0.001	3	4.07	0.010	14.50	NEAtl06>Med05

Table 6.8. Classification matrix of four albacore samples based on the three microchemistry parameters, CoreMg^{26} (log_{10}), CoreSr^{86} and EdgeSr^{86} (inverse).

	NEAtl05	NEAtl06	Med05	Med06	%correct
NEAtl05	9	3	5	1	50
NEAtl06	2	14	1	5	64
Med05	3	1	11	2	65
Med06	0	2	2	14	78
Total	14	20	19	22	64

Table 6.9. Jack-knifed classification matrix of four albacore samples based on the three microchemistry parameters, CoreMg^{26} (log_{10}), CoreSr^{86} and EdgeSr^{86} (inverse).

	NEAtl05	NEAtl06	Med05	Med06	%correct
NEAtl05	9	3	5	1	50
NEAtl06	5	11	1	5	50
Med05	3	1	11	2	65
Med06	0	2	2	14	78
Total	17	17	19	22	60

Table 6.10. Canonical discriminant functions of four albacore samples based on the three parameters of CoreMg^{26} (log_{10}), CoreSr^{86} and EdgeSr^{86} (inverse).

	1	2	3
CoreMg^{26} (log_{10})	-0.672	0.513	0.581
CoreSr^{86}	-0.017	0.995	-0.469
EdgeSr^{86} (inverse)	0.747	0.649	0.421

Table 6.11. Canonical scores of group means of four albacore samples based on the three parameters of CoreMg^{26} (log_{10}), CoreSr^{86} and EdgeSr^{86} (inverse).

	1	2	3
NEAtl05	-0.285	0.434	-0.233
NEAtl06	1.241	0.347	0.089
Med05	-1.363	0.264	0.155
Med06	0.056	-1.107	-0.022

Table 6.12. Classification matrix of four albacore samples based on the two otolith shape variables, circularity (adjusted) and C23.

	NEAtl05	NEAtl06	Med05	Med06	%correct
NEAtl05	5	4	4	5	28
NEAtl06	1	11	6	4	50
Med05	2	3	8	4	47
Med06	4	5	2	8	42
Total	12	23	20	21	42

Table 6.13. Jack-knifed classification matrix of four albacore samples based on the two otolith shape variables, circularity (adjusted) and C23.

	NEAtl05	NEAtl06	Med05	Med06	%correct
NEAtl05	5	4	4	5	28
NEAtl06	2	11	5	4	50
Med05	2	3	8	4	47
Med06	4	5	2	8	42
Total	13	23	19	21	42

Table 6.14. Canonical discriminant functions of four albacore samples based on the two otolith shape variables of circularity (adjusted) and C23 (- indicates F to remove statistic <0.4).

	1	2
Circularity (adjusted)	0.628	0.808
C8	-	-
C23	0.656	-0.786
C70	-	-

Table 6.15. Canonical scores of group means of four albacore samples based on the two parameters of circularity (adjusted) and C23.

	1	2
NEAtl05	-0.499	-0.166
NEAtl06	0.683	0.133
Med05	0.014	-0.590
Med06	-0.331	0.532

Table 6.16. Classification matrix of four albacore samples based on the four parameters of $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeSr^{86}$ (inverse) and C23.

	NEAtl05	NEAtl06	Med05	Med06	%correct
NEAtl05	10	2	4	2	56
NEAtl06	3	14	1	4	64
Med05	2	2	12	1	71
Med06	0	2	2	14	78
Total	15	20	19	21	67

Table 6.17. Jack-knifed classification matrix of four albacore samples based on the four parameters of $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeSr^{86}$ (inverse) and C23.

	NEAtl05	NEAtl06	Med05	Med06	%correct
NEAtl05	10	2	4	2	56
NEAtl06	4	13	1	4	59
Med05	4	2	10	1	59
Med06	0	2	2	14	78
Total	18	19	17	21	63

Table 6.18. Canonical discriminant functions of four albacore samples based on the four parameters of $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeSr^{86}$ (inverse) and C23 (- indicates F to remove statistic <0.4).

	1	2	3
$CoreMg^{26}$ (log_{10})	-0.557	0.690	-0.143
$CoreSr^{86}$	0.047	0.687	0.844
$EdgeSr^{86}$ (inverse)	0.839	0.514	0.126
Circularity (adjusted)	-	-	-
C8	-	-	-
C23	0.220	0.632	-0.738
C70	-	-	-

Table 6.19. Canonical scores of group means of four albacore samples based on the four parameters of $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeSr^{86}$ (inverse) and C23.

	1	2	3
NEAtl05	-0.320	0.153	0.586
NEAtl06	1.364	0.377	-0.122
Med05	-1.220	0.723	-0.315
Med06	-0.195	-1.297	-0.140

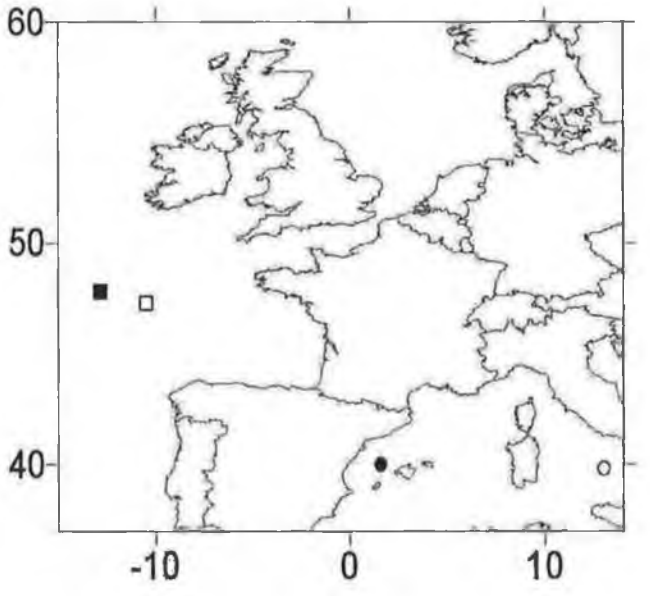

Figure 6.1. Sampling locations of albacore tuna; NEAtl05 (■), Med05 (●), NEAtl06 (□) and Med06 (○).

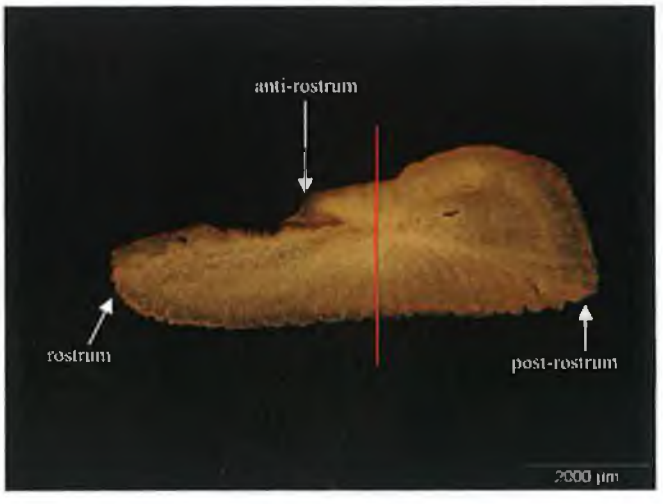

Figure 6.2. Position of the crop taken through the core (position indicated by red line) during image processing as depicted on an image of the internal face of a left whole otolith.

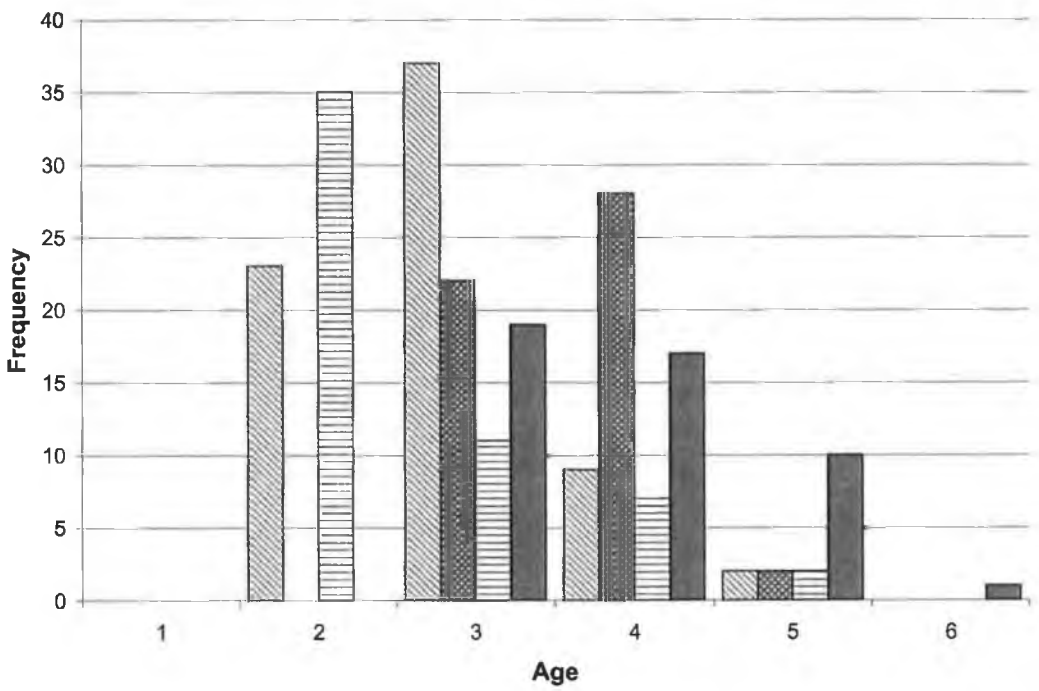

Figure 6.3. Age frequency distribution of albacore from four samples NEAtl05 (diagonal stripes), Med05 (spots), NEAtl06 (horizontal stripes) and Med06 (solid).

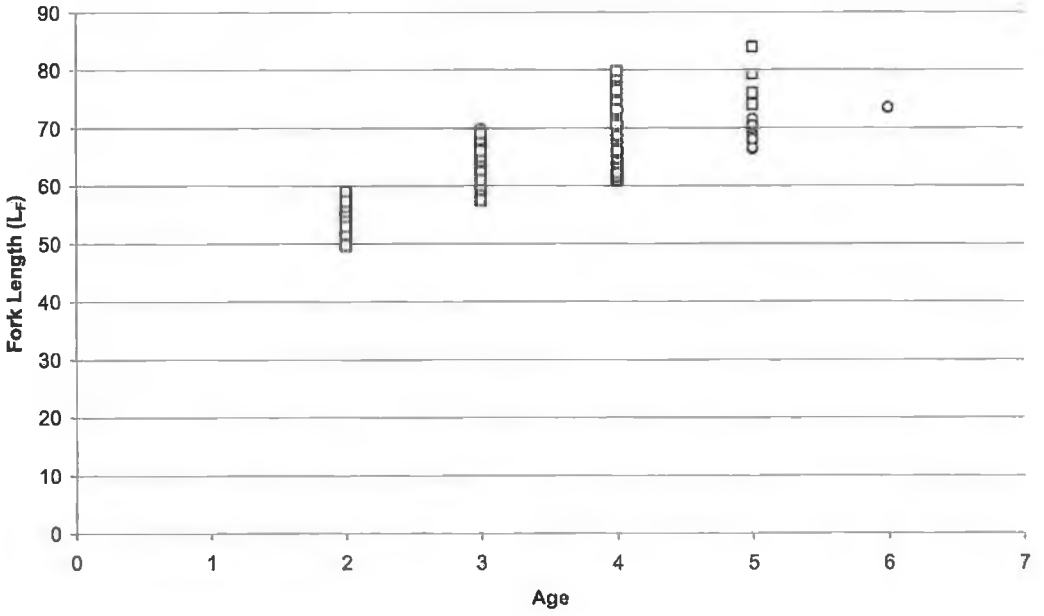

Figure 6.4. Mean fork length (L_f) at age of albacores taken from NEAtl (□) and Med (○).

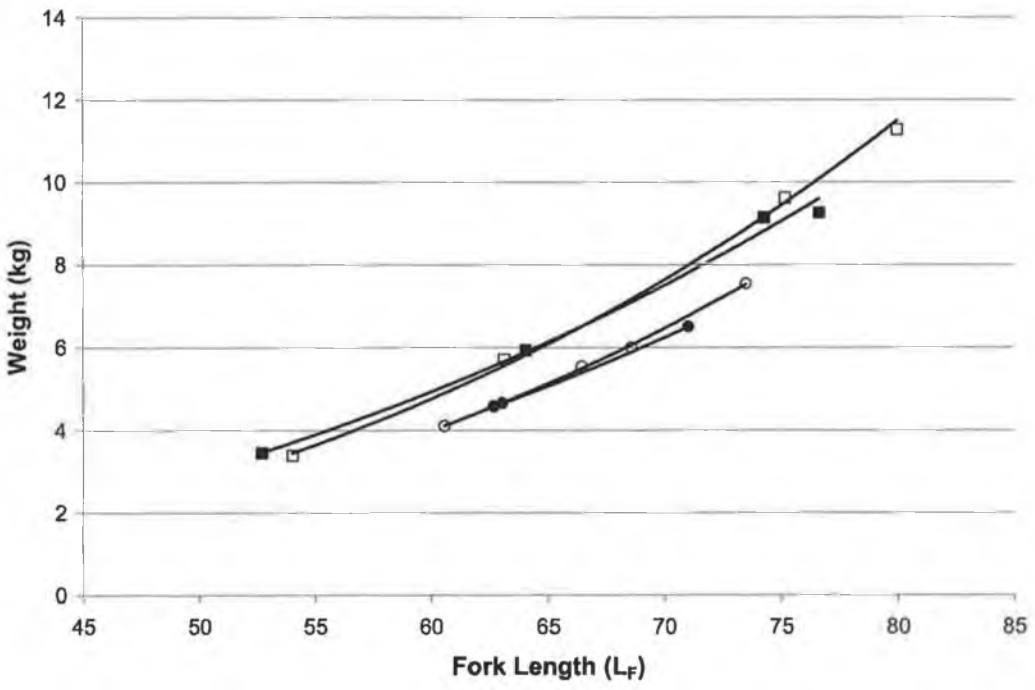

Figure 6.5. Mean weight at length of albacore by ages for NEAtl05 (■), Med05 (●), NEAtl06 (□) and Med06 (○).

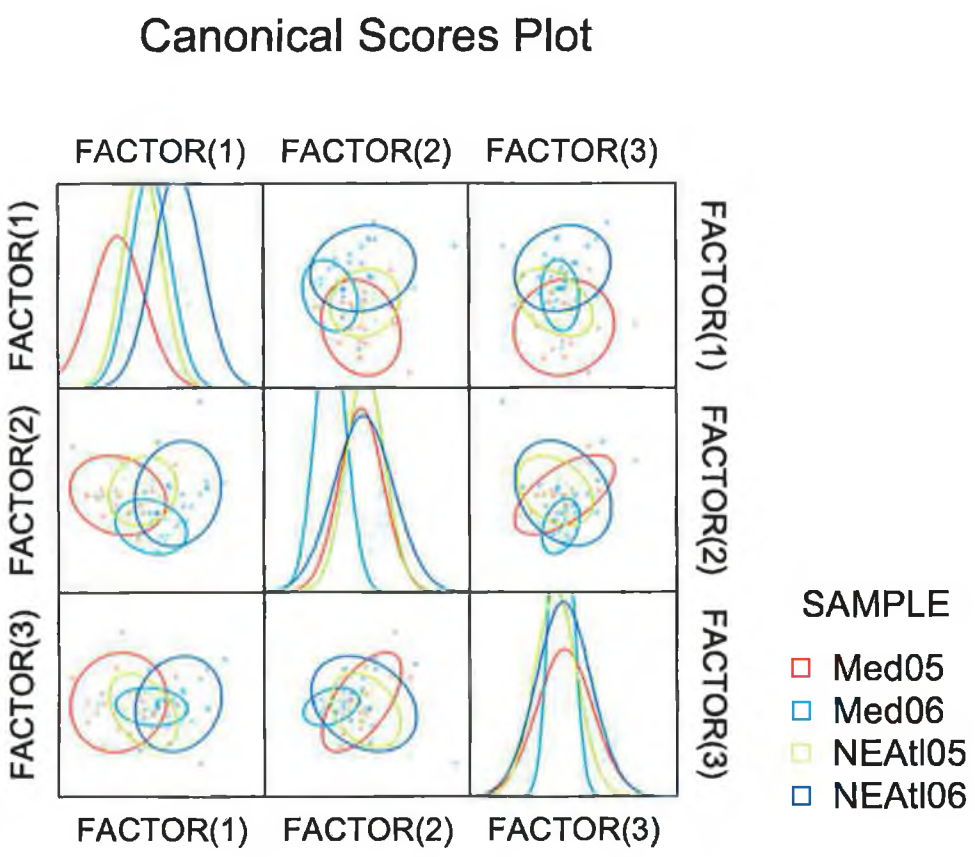

Figure 6.6. Canonical score plot for four albacore samples based on the three parameters of $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$ and $EdgeSr^{86}$ (inverse).

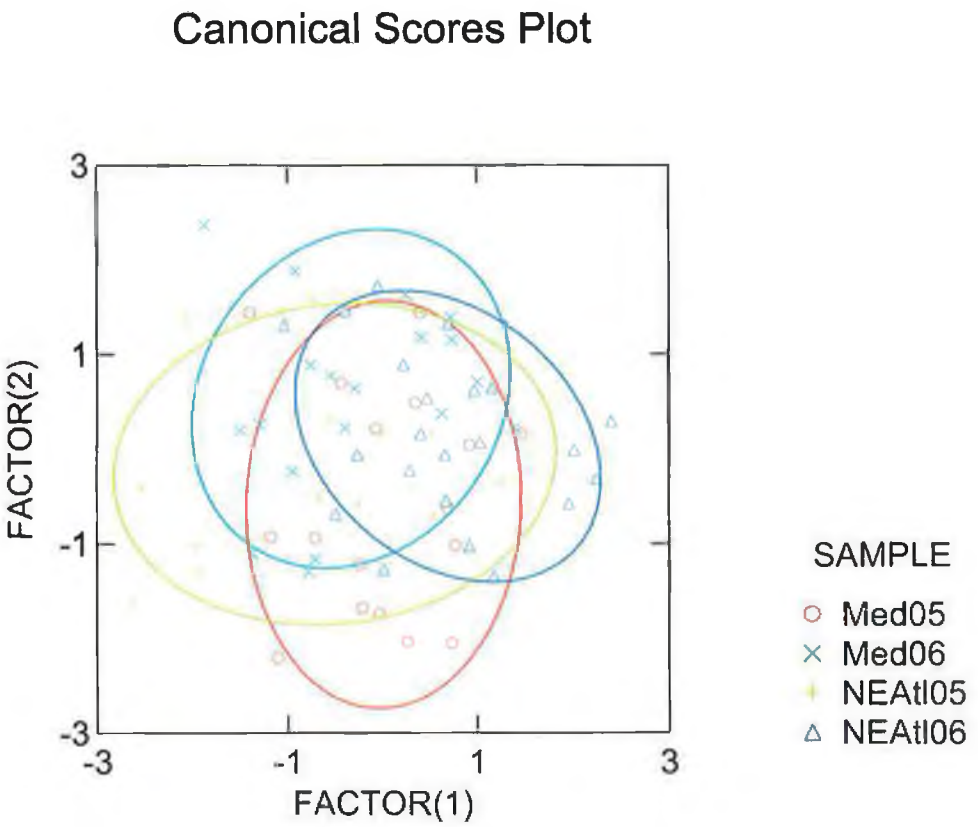

Figure 6.7. Canonical score plot for four albacore samples based on the two parameters of circularity (adjusted) and C23.

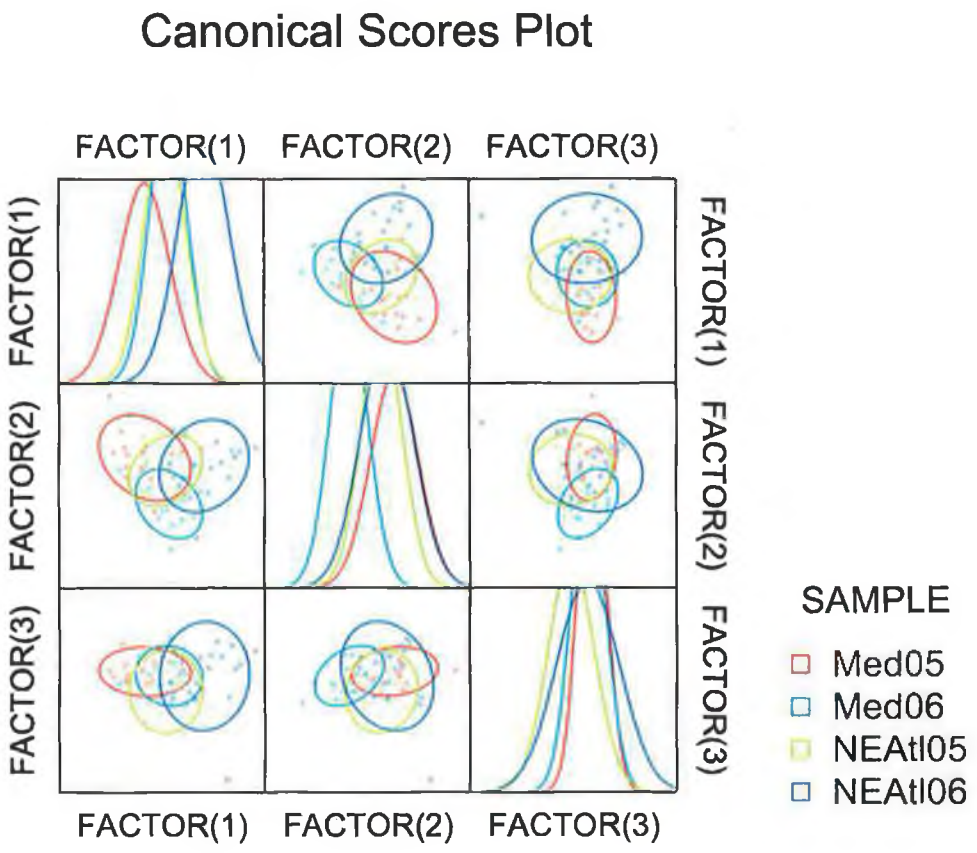

Figure 6.8. Canonical score plot for four albacore samples based on the four parameters of $CoreMg^{26}$ (log_{10}), $CoreSr^{86}$, $EdgeSr^{86}$ (inverse) and C23.

Chapter 7: General Discussion

7.1. Overview of management issues

Albacore tuna is a heavily exploited species of fish (Miyake *et al.*, 2004). Annual catches in the North Atlantic often exceed maximum sustainable yield (MSY) (Anon, 2008). ICCAT stock assessments state that if total allowable catch (TAC) repeatedly exceeds 30,000t the stock may not recover from being overfished (Anon, 2009). Management advice recommends reducing the number of vessels fishing to average numbers fishing between 1993-1995 with a reduced total allowable catch (TAC) (Anon, 2009).

Past fisheries management based on poor or inaccurate life history data and overestimates of the TAC accompanied by intense fishing pressure has led to the collapse of fishery stocks throughout the world. Examples of this include North Atlantic cod (Myers *et al.*, 1996) and orange roughy stocks in the South Pacific (Clark *et al.*, 2000). Using data which is deficient for fisheries management, or overly optimistic abundance estimates can have large ramifications not only on the species but also on the socio-economic status of the fishing and associated industries (Hilborn, 2007). Fisheries management must not only make decisions based on biological data but also on economic parameters, such as the economic sustainable yield (ESY) of a stock. If the ESY is greater that the MSY, then stock crash often follows, as shown in the case of the North Atlantic cod (Myers *et al.*, 1996).

The life history parameters of albacore are currently poorly known (Santiago and Arrizabalaga, 2005), and more data needs to be gathered on the environmental, phenotypic and genotypic aspects which contribute to differences in life histories. To determine sustainable exploitation rates and to ensure that appropriate management is applied to the North Atlantic and Mediterranean albacore stocks appropriate information must be made available to fisheries managers and decision makers.

Albacore tuna is a popular fish sold either as fresh steaks or tinned. Due to increasing public awareness of environmental issues the provenance and environmental sustainability of wild or farmed fish is becoming more important in consumer choice. There are several non-governmental organizations (NGO) which reward sustainable fishing with "eco-labels". The marine stewardship council (MSC; www.msc.org) is one of these global organizations which offered accreditation of sustainability to global fishing operations. There are a number of albacore fisheries worldwide that are marketed with an eco-label – e.g. the American Albacore Fishing

Association Pacific albacore fishery. The Irish albacore troll fishery is currently undergoing a similar assessment of sustainable fishing certification from another environmental sustainability scheme under the Friends of the Sea (www.friendsofthesea.org) friendly fishing scheme (Cosgrove, 2008). As sustainability is a commodity that can be marketed there is an increased economic incentive to address knowledge gaps in fisheries.

There are many challenges facing the sustainable management of fisheries (Dankel *et al.*, 2008). TAC of albacore is set by ICCAT on a biannual basis (Anon, 2009), however TAC can be difficult to regulate as albacores are targeted by multiple nations using a variety of different fishing methods and are landed in many different nations. Albacore are also exploited at both the juvenile and adult phases. This can lead to both recruit and growth overfishing. Albacore occupies a similar niche to bluefin tuna and is often subject to by-catch in the bluefin tuna fishery (ICCAT, 2003; 2007). Although biannual assessments of maximum sustainable yield (MSY) and total catch are conducted, there is a lack of information, especially fish dependant data, of the state of the Atlantic albacore stocks which has caused concern for the management committee at ICCAT (Anon, 2009).

7.2. Age and growth

Stock assessment of North Atlantic albacore uses growth parameters and age-length keys derived using direct estimates of age in combination with a length-based (ICCAT, 2007). Dorsal spines are the favoured structure for age estimation in albacore due to ease of access and minimal visual impact on the fish (ICCAT, 2003). However uncertainty remains in ageing albacore using the spines. This study has shown that there are inconsistencies associated with age estimates obtained from spines scales and otoliths. Although scales were proposed by Megalofonou *et al.* (2003) as a potential alternative to spines for ageing of Mediterranean albacore, they were found in this study to have many disadvantages; indistinct annuli and calcification of older scales often made them impossible to read. Otoliths showed similar problems with poor contrast between opaque and translucent increments and confusion occurring due to the presence of annual couplets (Compean-Jiménez and Bard, 1983). Mean CV of age estimations using spines was high (20-27%) with low

percentage agreement between the six age readers (25 to 39%). There are also reports of ambiguity surrounding the interpretation of multiple banding in spines (Compean-Jiménez and Bard, 1983; Oritz de Zárate *et al.*, 2007a). Therefore, age estimations in albacore can be highly age-reader dependant. This can have serious implications for the management of the stock, especially when many age-readers contribute data towards the age based model that is used in making management decisions.

In order to improve precision of age estimations more inter laboratory exchanges and collaborative exercises are required, these should also be undertaken on a frequent basis, potentially ever four years to link with the ICCAT albacore assessment sessions. Spine structures are collected on an annual basis for management and academic research, and presumably large quantities of archived material are available throughout institutions globally (i.e. IFREMER, France; AZTI and IEO, Spain; BIM, Ireland; CSIRO, Australia; Secretariat for the Pacific, New Caledonia), these samples could be made available to undertake such exchanges and collaborative exercises.

Accuracy is defined as the comparison between age estimation and the true age of a fish (Kimura and Lyons, 1991), whereas precision is the percentage agreement or variability between readings. Accuracy of age estimation of albacore can be resolved through validation of increment deposition by tagging fish with oxytetracycline (OTC), which marks the calcareous structures which is visible under ultraviolet light. OTC tagging surveys have been carried out on albacore (Oritz de Zárate *et al.*, 1996; Metrio *et al.*, 1997) and other tuna species (Hallier *et al.*, 2005)(Stockwell, pers. comm.) for age validation. Tagging experiments are fraught with difficulties; they are dependant on the survival of fish after tagging, on the returns of tagged fish by fishermen and of the biological material by researchers in other institutions. BIM undertook a tagging exercise between 2005 and 2007 (Cosgrove *et al.*, 2006); only five recaptures were reported (2.5%), and calcareous structures were returned for just three of these fish (1.5%), despite the additional financial incentive offered for the whole fish (€200 per fish). ICCAT recommends undertaking large-scale well designed tuna tagging programs in the Atlantic which are comparable to similar studies underway in other tuna commission areas (i.e. IOTC, IATTC and WCPFC) (Anon, 2009). In order to ensure success in any future tag-release studies, which are necessary for assessing the basic information on stocks and fishery status (Anon, 2009), it is necessary to ensure that there are sufficient tagging

returns, i.e. large numbers of fish need to be tagged (over 100,000 tunas were tagged in the Indian Ocean between 2003 and 2008, (Stockwell, pers. comm.)). This would be a huge undertaking in the Atlantic Ocean which requires international and cross-institutional co-operation.

In this study growth differences were observed between yearly cohorts of albacore. The parameters currently used is based on age estimated from Bard (1981) (ICCAT, 2007), it is possible that growth rates may have changed with fishing and environmental pressures over time; therefore a new assessment of growth rates should be undertaken for the North Atlantic, South Atlantic and the Mediterranean stocks. Strategic sampling through the recommended ICCAT scientific observer and logbook programs (Anon, 2009) of albacores should be undertaken on a regular basis throughout the fishing season in the North East Atlantic (July to November) in all ports where albacore are landed, and throughout the year in the adult portion of the fishery to gather information on cohort specific growth. These programs are generally funded by regional or national governments, or through scientific institutions, although ICCAT does not make any recommendations as to the current source of funding for these proposed programs (Anon, 2009). Sampling for fishery information needs to be consistent with a co-ordinated sampling effort and consistent protocols across all areas. In addition to parameters proposed by Oritz de Zárate *et al.* (2007a) it is suggested that information on fish condition (i.e. weight and circumference of the fattest point of the fish) should be gathered routinely. This information should be analysed in combination with other factors, such as food availability and environmental conditions such as water temperature, to produce stock abundance models more appropriate to the current state of the albacore stocks.

It is possible that the age based model used for albacore stock assessments should incorporate cohort specific growth. Variation in cohort growth rate can be affected by ambient water temperature (Chiu and Chen, 2001; Kikkawa and Cushing, 2001) food availability and type (North *et al.*, 1998; Mikheev and Wanzenböck, 1999). A particularly strong cohort (either in number and size or reproductive output) in the fishery can create peaks in abundance (Chen and Mello, 1999). Estimating greater stock abundance from strong cohorts can have implications for the stock; strong cohorts may be followed by weaker ones which may be heavily exploited based on the data from previous cohorts (Hilborn and Walters, 1992). This illustrates the sensitivity of fisheries production to life history traits of growth, survival and

fecundity (Wootton, 1999). Therefore stock assessments need to be regularly updated and calibrated to incorporate recent trends in the fisheries (Chen and Mello, 1999; Bjørnstad *et al.*, 2004; Chen *et al.*, 2008).

Fish stocks are not closed units but are affected by a wide variety of factors. Relatively little is known about the life history parameters of albacore (Santiago and Arrizabalaga, 2005) with respect to behaviour, feeding, environmental influences on growth, migration, etc. Climate change is a factor which potentially will affect all global fisheries (Roessig *et al.*, 2004). Presently we know little about how resilient this stock would be to large scale environmental changes (Bard, 2003; Arregui *et al.*, 2006). The Oceanic Fisheries and Climate Change project (OFCCP GLOBEC) are currently investigating the effect of climate change on the productivity and distribution of oceanic tuna stocks and fisheries in the Pacific Ocean (www.spc.int); this could inform the development of similar programs for tuna stocks in the Atlantic.

Fisheries management is currently moving towards "the ecosystem approach"; this approach relies on the principle that ecosystems have a natural resilience (Frid *et al.*, 2006). The challenge for management is recognising key limits, such as the types and levels of anthropogenic activities that can be sustained without compromising the functionality of the ecosystem. The ecosystem approach relies on the understanding of ecosystem structure, processes, functions and interactions but is also dependant on sound political strategies necessary to obtain ecosystem balance (Frid *et al.*, 2006). (Lehodey *et al.*, 2008) have developed a model specifically for tuna (SEAPODYM) using data from a wide variety of different ecosystem habitats and population dynamics to model abundances over a fine spatial scale; this model may prove useful for modelling albacore abundances over a similar scale in the North Atlantic and Mediterranean Sea. However as very little is known about the ecosystem interactions of this key predator, it is therefore difficult to anticipate the effect that different models will have on overall management of the stock (Mackinson *et al.*, 2009).

7.3. Stock structure

Microsatellite DNA data showed conclusively that the North Atlantic and Mediterranean fish are distinct and this is was expected as limited exchange has been observed between these stocks (Arrizabalaga *et al.*, 2002; Arrizabalaga *et al.*, 2003).

This supports the separate management of the North Atlantic and Mediterranean stocks (ICCAT, 2007). However heterogeneity was observed within both areas, this is not currently a consideration in stock management (ICCAT, 2007). The existence of discrete groups within exploited stocks is a parameter which should be considered in fisheries management. If equal fishing pressure is applied to stock components with differences in biomass and resilience, it could lead to the potential collapse of the less productive population (Hutchinson, 2008). This can lead to local extinctions of stocks and potentially the eradication of fine scale variation in functional genes which may allow species to adapt to altering conditions caused by climate change (Carvalho and Hauser, 1994; Hutchinson, 2008).

Bluefin tuna is a closely related sympatric species to albacore tuna which has been studied in greater depth, partly due to its higher commercial value. Two separate components of bluefin have been observed in the North Atlantic with overlapping geographical distributions. The stock appears to be patchy in distribution (Rooker *et al.*, 2007) and this creates issues for effective management; the western stock is vulnerable to overfishing when both stocks mix in the central Atlantic. Rijnsdorp *et al.* (2007) suggest that TAC management, although successful in some single-species fisheries, leads to the unsustainable exploitation of mixed stocks. It is proposed that total allowable effort (TAE) is used as a more efficient management measure; this takes into account the differences in catch efficiency between fleets and seasonal changes in the distribution of the target species (Rijnsdorp *et al.*, 2007). Rooker *et al.* (2007) suggest spatially explicit management or temporal closures of the bluefin fishery when the level of mixing of stock components is known to be very high. To propose similar management schemes for North Atlantic albacore, the structure of the stock must be determined more conclusively.

Further tagging studies of albacore could provide useful information with regards to the proposed heterogeneity in the North Atlantic stock (Hue, 1979; 1980). In the most recent studies using tag-recapture, returns have ranged between 1 to 7%, with an average of 3% returns (Arrizabalaga *et al.*, 2004). On this basis in order to obtain sufficient returns to make a study viable thirty times more fish must be tagged than the number required for analysis. Tagging studies incur great expense, however they may provide valuable information regards migration movements of fish in the stock. Pop-up satellite archival tags have been utilized on large fish such as bluefin tuna and basking sharks to great effect (Sims *et al.*, 2003; Sibert *et al.*, 2006), these

tags have been trialled in albacore tuna with limited success (Cosgrove, 2008); improvements in the technology i.e. the development of smaller pop-up archival tags and increased post-operative survival rates of albacores may mean that this method of tagging would be of great value in future studies.

If accurate migration patterns could be ascertained from tagging surveys then studies targeting spawning aggregations and nursery grounds could be undertaken. Investigations of the larval distribution and nursery grounds could be combined with survey on other species undertaken by other institutions to spread the survey costs, such as an investigation into the migration and spawning of the European eel, *Anguilla anguilla* (Hanel, pers. comm.). Genetic population studies in fisheries usually obtain samples from discrete spawning areas (Jørgensen *et al.*, 2005; Was *et al.*, 2008) and information from the spawning stock component can be used to estimate a minimum genetic effective population size (O'Connell and Wright, 1997) which can be compared to stock biomass and MSY. A microsatellite DNA study on both adult and larvae would ascertain whether separate components exist within the spawning grounds. If differences are observed, it may be possible to attribute these potential differences to environmental variables or landscape genetics (Manel *et al.*, 2003), further adding to our understanding of the North Atlantic albacore stock.

7.4. Otolith chemistry and shape

This study has provided new information on otolith chemistry and otolith shape. It has been shown that calcareous structures are susceptible to contamination by external sources; however this contamination can be removed using a nitric acid wash. Otolith chemistry indicated small differences in the core region of sectioned otoliths from fish sampled in the North Atlantic and Mediterranean. Contamination does not seem to have been an influencing factor in the small differences between samples obtained from the two regions as the elements used in the core and edge chemical analysis were shown to be resilient to contamination. However contamination effects may mask potential group differences.

Genetic distinction was evident between the 2005 and 2006 North Atlantic samples which suggest the possibility of distinct larval origins. However, the environmental differences in core chemistry showed insufficient variation to be able

to distinguish between these two samples. Edge chemistry was used to distinguish between genetically distinct samples; however as the information gathered during the last few months of the fish's life is likely to be attributed to the ambient conditions, this information does not add greatly to stock separation.

Insufficient differences in the otolith shape descriptors were present to distinguish between North Atlantic and Mediterranean albacore. As limited variability was shown within stocks using information gathered from otoliths, it draws into question the benefits of using otoliths for stock description and age assessments in albacore tuna.

Otoliths from tunas are hard to obtain and often researchers must purchase the whole fish to obtain this small structure. ICCAT in Anon (2009) recommend the collection of representative samples of bluefin tuna otoliths as these are good indicators of stock origin; however this study has shown that otoliths are not as valuable in albacore stock assessments. Information from microsatellite DNA is relatively expensive (approximately €10 to €15 per sample for 6 microsatellite loci) it has been shown to be a more valuable descriptor of albacore stock heterogeneity than any of information gathered from otoliths by age, shape or chemistry. Fish often need to be purchased at market value in order to obtain otoliths, which makes the cost of purchasing albacores for the otoliths more expensive than the cost of DNA analysis per fish. As dorsal spines are the preferred ageing structure, it is proposed that future studies move away from using otoliths in albacore stock structure studies and concentrate on genetic variability. Tissue for DNA can easily be obtained during routine sampling with the removal of the dorsal spine.

7.5. General Conclusions

There are several major findings within this work which add to the overall knowledge of albacore tuna and also contribute towards the knowledge of specific methods used in stock analysis.

Firstly there are issues with the ageing of individual fish; substantial potential bias was observed among readers regardless of experience and between the different calcareous structures used for ageing. Relatively low precision was also observed in repeated age estimations by one reader using the same structure. This shows that the

age estimations of a single reader using one structure cannot be relied upon as definitive, when this data is used for catch at age and length at age matrices which are used as the basis for modelling information required for making management decisions. It has also been shown that it is easier to obtain agreement between age readings of albacores of Mediterranean origin. This may be due to unknown physiological differences which are manifested in the deposition of the calcareous structures as they grow throughout the fish's life.

It can be concluded from this study that there are differences in growth rates of albacore from North Atlantic and Mediterranean origin as previously suggested by Megalofonou (2000). Also there are substantial differences in the weight to length ratios of fish originating in these two ocean basins. There is also the suggestion of enhanced survival of faster growing fish. With this in mind the back calculation models incorporating cohort, age and size effect are the most informative for albacore and should be utilised in the current ICCAT stock assessment model. Although no estimation of stock abundance has ever been conducted for albacore in the Mediterranean, it has been shown here that if such an assessment were carried out, new parameters for Mediterranean fish would need to be calculated and that it is not suitable to apply the same parameters to Mediterranean fish as are applied in the assessment of North Atlantic albacores.

This study has shown that contrary to previous scientific understanding, otoliths (and also dorsal spines) are subject to internal contamination following sectioning and use in probe-based analyse. This can have consequences for interpreting past probe based microchemical studies. It has also been shown that translucent slower growing increments were more susceptible to contamination, this result can be used to assist the development of future probe based microchemical studies, where contaminating effects may compound the "true" results of the study. It has been shown that the most effective method of removing surface contamination from sectioned otoliths and dorsal spines was by using 5% nitric acid; however care must be taken not to damage the test material by over exposure to the acid. It has also been shown that some contaminants cannot be removed from the calcareous structure; therefore care must be taken in future studies to ensure that misleading results are not used as the basis for stock delineation. As well as showing that certain areas of the otoliths and spines are more susceptible to contamination it has also been shown that the internal microchemistry of otoliths and dorsal spines are not homogenous, but

rather are dependant on the internal physiology of the structure. It has also been definitively shown that the microchemistry of otoliths and dorsal spines are not correlated adding further evidence to the differences in metabolic pathways used in the deposition of these different calcareous structures. Although some differences were observed between the microchemistry of albacores from North East Atlantic and Mediterranean origin, the ability to classify fish using this particular method is lower than would be expected for definitive stock classification. However other probe based analyses such as stable isotopes may prove more effective in classifying albacores to region of origin.

Otolith shape analysis although effective in other species of fish, has shown to be a poor indicator of stock origin in albacores. An analysis of whole otoliths rather than crops may prove more useful in stock discrimination, however with the logistical problems associated with firstly obtaining otoliths and secondly extracting and transporting them without damage, may make this method of stock description unviable in future studies of albacore tunas.

It has been shown in this study that microsatellites developed for bluefin tuna, a sympatric species of the same genera, successfully amplify in albacore tunas. However several differences were observed in the number of alleles, heterozygosities and potential of null alleles between the two species; the differences indicated that basing a microsatellite DNA study on the published characteristics of bluefin tuna is not appropriate. The genetic markers have shown for the first time that there is a difference in stock composition between the western and central Mediterranean albacore stock. It can be concluded from this that genetic heterogeneity is evident in a stock that was previously thought to be homogenous. It is possible that this is a manifestation of the spatial discontinuity observed in albacores within the Mediterranean Sea basins. Management of this stock may have to be revised to recognise the distinct genetic populations which contribute to the overall stock and to ensure that overexploitation of one contributing population does not occur. It has also been shown that there is temporal stability in the genetics of albacore within the central Mediterranean fishery. Although less definitive than the differences observed in the Mediterranean, there is evidence of heterogeneity within North Atlantic albacore, based on feeding aggregations, which are fished along the Porcupine Ridge. This may have implications for fishery management where the effective number of individuals contributing to the overall production may be skewed due to previously

unknown sub-populations. Some researchers (Bard, 2003) have indicated that the North Atlantic stock is losing its resilience to fishing pressure, the loss of a genetic component or sub-population may further exacerbate this loss and contribute towards the crash of the fishery at large.

Of all the methods trialled in this study, genetic analyses based on microsatellites provided the best discriminatory power for assigning individuals to either North East Atlantic or Mediterranean origin compared to elemental analysis, otolith shape and growth indices. This indicates that a multi-generational lineage marker (or allopatry) is more effective at showing the differences between and within the two stocks when compared to the markers which indicate differences over shorter time-scales, such as individual growth and otolith chemistry.

7.6. Recommendations for Future Management

The management of highly migratory species, which are targeted by a multitude of nations, such as albacore tuna produces a variety of problems for fishery managers. The current management of albacore tuna in the North and South Atlantic Ocean and the Mediterranean Sea is conducted by the International Commission for the Conservation of Atlantic Tunas (ICCAT).

Based on the results from this study, I would recommend that ICCAT need to undertake a comprehensive stock analysis of albacores in the Mediterranean Sea, since a stock analysis of Mediterranean albacore has never been conducted (ICCAT, 2009). A traditional study based on morphometric characteristics and ageing profiles of the albacore found in both the eastern and western basins should be conducted in conjunction with a microsatellite DNA study of these fish, to better understand the discontinuous distribution shown by albacore in Mediterranean Sea, with suitable total allowable catch (TAC) limits set on the basis of effective population sizes and recruitment potential of the population or sub-populations within the Mediterranean basins. This is especially pertinent in light of new management protocols which have been imposed for bluefin tuna, such as closing of the bluefin fishery from May to June (URL 2). This may put additional fishing pressure on albacores, as there is no total allowable catch in the Mediterranean Sea (ICCAT, 2009). The use of genetics should be utilised further to distinguish the discrete sub-populations of albacore in the

Mediterranean ensuring that any one population is not overexploited, especially as the region where the fish was caught may not be in the same basin as where the fish is landed. This method can also be used as a tool for forensic genetics in the management of Mediterranean tunas; the two species of tuna, albacore and bluefin, are morphometrically very similar and if the pectoral fin is removed, then it is only possible to distinguish the two species by genetic analysis. This method may prove effective in preventing misreporting of catches or selling one species under the guise of another by unscrupulous members of the fishing community.

In the North Atlantic, management is based on total allowable catch which is derived at stock assessment meetings every four years (URL 2). However the stock assessments are currently based on data which is nearly 20 years old (ICCAT, 1990), this data may be valid today, however further study is required. To be able to discount growth overfishing in the North Atlantic, further assessments of growth should be undertaken on a frequent basis to coincide with the stock assessment meetings every four years. It would also be proposed that the management of potential genetic heterogeneity or different migratory components of the stock be recognised by managers. If separate trophic migrations are taking place, which in this study are show differences in genetic characteristics then instead of having a single total allowable catch for the entire year, a TAC should be enforced per month during the fishing season between June and November, and once the total allowable catch for the month has been reached, the fishery should be closed, and no further fish landed. These proposals may be met with resistance from the fishing community, however stricter management must be enforced to ensure the sustainability of the North Atlantic and Mediterranean albacore stocks. This is important for future generations especially when the general life history is poorly understood and the stock is losing its resilience to current fishing pressures (Bard, 2003).

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"My strength lies solely in my tenacity"

Louis Pasteur