

**Universidade de Lisboa  
Faculdade de Ciências  
Departamento de Biologia Animal**

**The University of Adelaide**



**THE UNIVERSITY  
*of* ADELAIDE**

**Assessing connectivity in coastal systems  
as a tool for fish management and conservation**

**Patrick Neil Bowskill Reis dos Santos**

**Doutoramento em Biologia  
Especialidade de Biologia Marinha e Aquacultura  
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as a tool for fish management and conservation**

**Patrick Neil Bowskill Reis dos Santos**

**Tese orientada pelo Professor Auxiliar com Agregação Henrique Nogueira  
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**Patrick Neil Bowskill Reis dos Santos**

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## Abstract

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Connectivity is a critical component of marine fish populations as it drives population replenishment, determines colonization patterns and populations' resilience to harvest. Knowledge of individual movement patterns is pivotal to ascertain population dynamics and underpins sustainable management. Many marine fish species occupy spatially segregated habitats throughout their life history. Estuarine nursery function and the ecological link between estuaries and the coastal environment were reviewed with emphasis on quantifying estuarine contributions and assessing fish movement to coastal adult populations. The chemical composition of otoliths is one of the most promising natural tags to quantify fish movement, particularly for early life stages. Yet, many uncertainties remain regarding the factors affecting elemental incorporation in otoliths. Temperature, salinity and water composition variations were examined to evaluate their interactive effects on *Dicentrarchus labrax* otolith chemistry, which is a vital first step in reconstructing environmental histories and intra-estuarine movement patterns. The combined use of otolith chemistry and muscle stable isotopes provided corroborative and complementary information, at different spatio-temporal scales, of intra-estuarine habitat use and outlined limited movement between segregated estuarine nursery areas. Otolith elemental signatures of juvenile *Platichthys flesus* and *D. labrax* collected in estuarine nurseries along the Portuguese coast were year specific; though variation among seasons did not hinder spatial discrimination. Juveniles were accurately assigned to their estuaries of origin. These elemental signatures constituted the baseline for assessments of connectivity between juvenile and adult populations. The quantification of the relative contributions of estuaries to coastal populations of *P. flesus* and *D. labrax* in two years enabled the identification of the estuarine systems which contributed most to the replenishment of coastal populations. Overall, results contributed to prioritization of the conservation of ecologically important habitats and to the development of integrated management strategies for these commercially important species.

**Keywords:** Connectivity; natural tags; otolith chemistry; estuaries; nurseries



## Resumo

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A conectividade é uma componente crítica das populações de peixes marinhos na medida em que determina a manutenção das populações, os padrões de colonização e a resiliência à pesca. O conhecimento dos padrões de movimento individuais é fundamental para avaliar a dinâmica populacional e promover uma gestão sustentável. Muitas espécies de peixes marinhos ocupam habitats segregados ao longo da vida. A função de viveiro e a ligação ecológica entre estuários e costa foi revista com ênfase na estimativa das contribuições estuarinas e movimentos de indivíduos para os mananciais costeiros de adultos. As assinaturas químicas em otólitos são um marcador natural notável para estimar movimento, sobretudo em estados de vida iniciais. Porém, permanecem muitas incertezas relativamente à influência dos factores ambientais na incorporação e composição química de otólitos. Foram examinados os efeitos da salinidade, temperatura e composição da água em juvenis de *Dicentrarchus labrax*, um passo essencial para o uso da química de otólitos para reconstruções ambientais e de movimentos intra-estuarinos. O uso integrado de assinaturas químicas de otólitos e isótopos estáveis no músculo forneceu informações complementares sobre a conectividade e movimento limitado entre áreas de viveiro distintas num estuário, a diferentes resoluções espaço-temporais. As assinaturas químicas de juvenis de *Platichthys flesus* e *D. labrax* variaram entre anos, e meses, mas a variação sazonal não confundiu a discriminação espacial. Os juvenis foram correctamente classificados aos seus estuários de origem e as assinaturas constituíram as bases de referência para avaliações de conectividade entre populações de juvenis e adultos. A quantificação das contribuições relativas das áreas de viveiro estuarinas permitiu identificar os estuários que mais contribuíram para a reposição das populações costeiras destas espécies nos dois anos analisados. Globalmente, os resultados obtidos contribuem para identificar e priorizar a conservação de habitats ecologicamente importantes e para o desenvolvimento de planos de gestão integrados para estas espécies.

**Palavras-chave:** Conectividade; marcadores naturais; microquímica de otólitos; estuários; áreas de viveiro



## Resumo alargado

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Os estuários e zonas costeiras são dos ecossistemas mais produtivos do planeta e o seu papel como áreas de viveiro para juvenis de numerosas espécies de peixes marinhos é amplamente reconhecido e alvo de diversos estudos motivados pelo interesse ecológico e económico destas áreas e recursos associados. Muitas espécies de peixes marinhos utilizam estuários e zonas costeiras para completar os seus ciclos de vida. Os juvenis destas espécies passam meses a anos nestes ambientes até recrutarem às populações de adultos ao longo da costa. A troca de indivíduos entre grupos geograficamente separados, ou conectividade, é uma propriedade fundamental dos ciclos de vida destas espécies com fases de vida segregadas, e tem um papel primordial na dinâmica populacional, colonização de novos habitats, e resiliência das populações ao impacto da pesca. Os estuários e as zonas costeiras constituem um elo vital entre os diferentes estados de vida, e assumem particular importância no recrutamento e contribuição de indivíduos para as populações de adultos ao longo da costa. Um melhor conhecimento dos padrões e taxas de movimentos de peixes entre diferentes habitats é fundamental para o desenvolvimento de estratégias de gestão e conservação dos recursos bem como para a identificação de habitats ecologicamente importantes. No contexto actual, de amplas alterações nas zonas costeiras por acção humana, com degradação ou perda significativa de habitat, e de um consenso generalizado relativamente à sobreexploração de muitos recursos pesqueiros, a aferição da conectividade em populações de peixes reveste-se de particular importância.

A quantificação e estimação do movimento individual e da conectividade entre populações de peixes é uma tarefa complexa, sobretudo para estados de vida iniciais, que ao longo do tempo foi sendo abordada através de diversas técnicas. Porém, a interpretação de diferentes marcadores naturais, com destaque para a análise de assinaturas químicas em otólitos de peixes, representa um avanço notável para determinar padrões e taxas de movimentos e quantificar conectividade em populações de peixes. Neste contexto, o presente estudo tem como objectivo principal avaliar a conectividade entre estuários e populações costeiras, com particular destaque para a utilização de assinaturas químicas de otólitos, estimar as contribuições relativas de áreas de viveiro estuarinas para as populações costeiras de adultos e caracterizar a sua variação espaço-temporal como instrumento essencial para o desenvolvimento de estratégias de gestão e conservação.

Esta tese é composta por sete capítulos, cinco dos quais correspondem a artigos científicos, produzidos para responder aos objectivos propostos, que estão publicados ou em revisão em revistas internacionais com arbitragem científica. Estes capítulos são precedidos de

um capítulo de introdução geral e seguidos de um capítulo de discussão geral que inclui também sugestões de desenvolvimentos futuros.

Na introdução geral (Capítulo 1) foi efectuado um enquadramento teórico do tema da presente tese focando a importância da avaliação de conectividade em populações de peixes e a identificação retrospectiva das áreas de viveiro de populações de adultos capturados ao longo da costa. Neste âmbito, a quantificação de conectividade entre populações é fundamental para a identificação de habitats importantes, para o conhecimento da estrutura dos mananciais costeiros, e para o desenvolvimento e implementação de medidas de gestão e conservação eficiente de habitats e recursos pesqueiros a escalas espaciais apropriadas. É dado particular destaque às assinaturas químicas de otólitos, enquanto marcadores naturais de movimento e de uso de habitat, e a questões relacionadas com a sua correcta aplicação, bem como ao seu potencial uso integrado com outros marcadores naturais, nomeadamente a análise de isótopos estáveis, em estudos de conectividade.

No capítulo 2 foi dado destaque à estrutura das comunidades de peixes estuarinos e às funções ecológicas desempenhadas pelos sistemas estuarinos, com particular ênfase na função de viveiro para juvenis de espécies de peixes com estados de vida segregados, na conectividade entre sistemas estuarinos e costeiros, e na sua importância para as pescas costeiras. Após uma reflexão crítica sobre o papel dos estuários como áreas de viveiro, e da evolução do conceito de áreas de viveiro, segue-se uma caracterização do desenvolvimento das metodologias para avaliação de movimento e conectividade ao longo das últimas décadas. É feita ainda uma revisão do estado da arte relativamente à conectividade entre juvenis em estuários e as populações costeiras de adultos, e é destacada a importância da quantificação da contribuição relativa de diferentes habitats para os mananciais costeiros. Por último, a informação recolhida é analisada na perspectiva do interesse da preservação da função de viveiro estuarina e da sua importância para uma gestão e exploração sustentável de recursos.

No capítulo 3 foi desenvolvida uma abordagem experimental para avaliar as relações entre a composição química dos otólitos de juvenis de robalo *Dicentrarchus labrax* e as propriedades físico-químicas da água (temperatura, salinidade e composição química). Os resultados foram analisados considerando a possibilidade de utilização das variações nas assinaturas químicas de otólitos para a reconstrução do uso de habitats estuarinos e identificação de habitats essenciais para juvenis desta espécie. Para a caracterização de uso de habitat e a reconstrução da história ambiental de peixes é necessário que se estabeleçam relações preditivas entre a composição química de otólitos e o meio ambiente circundante. No presente trabalho, foi descrita uma interacção entre a salinidade e a concentração de Sr:Ca e Ba:Ca na água, bem como uma relação positiva da incorporação e composição química dos otólitos com a temperatura. Os resultados evidenciam as relações entre a incorporação dos elementos e a sua respectiva disponibilidade no meio ambiente. Compreender de que forma os movimentos entre gradientes ambientais podem ser inferidos é fundamental para se poder estabelecer a relação entre a composição química dos otólitos de indivíduos capturados na natureza e a reconstrução da sua história ambiental, possibilitando a descrição retrospectiva dos seus

padrões de uso de habitat.

A análise da variabilidade espaço-temporal das assinaturas químicas de otólitos (Capítulo 4) é um pré-requisito essencial para usar a composição química de otólitos como marcador natural, nomeadamente para determinar as áreas de viveiro de origem de peixes adultos. Para além da confirmação da capacidade de discriminação espacial entre grupos de origem, à escala de interesse, é particularmente importante avaliar potenciais variações temporais nas assinaturas químicas, e a sua possível influência na discriminação espacial. A variação temporal pode ocorrer a diferentes escalas e confundir a discriminação espacial entre grupos de origem. As assinaturas químicas dos otólitos (Li:Ca, Mg:Ca, Mg:Ca, Cu:Ca, Sr:Ca, Ba:Ca, Pb:Ca) de juvenis de solha *Platichthys flesus* e robalo *D. labrax* recolhidos ao longo de dois anos em áreas de viveiro estuarinas ao longo da costa portuguesa revelaram variabilidade às escalas espaço-temporais amostradas. Em geral, os juvenis de ambas as espécies foram correctamente classificados aos seus estuários de origem. As variações mensais/sazonais tiveram pouca interferência na capacidade de discriminar entre estuários; porém, as assinaturas químicas revelaram elevada especificidade anual. As assinaturas químicas determinadas em juvenis de *P. flesus* e *D. labrax* constituem as bases de referência para a subsequente avaliação da conectividade entre populações de juvenis e de adultos para estas espécies.

No capítulo 5, foi avaliada a conectividade entre áreas de um estuário para *D. labrax* e para caboz comum *Pomatoschistus microps*. Esta é uma medida importante relativa ao uso funcional dos estuários, e é fundamental para o reconhecimento de habitats essenciais e ecologicamente importantes para peixes, bem como para averiguar as escalas espaciais em que devem ser implementadas medidas de protecção de habitats, tendo em vista, por exemplo, a preservação da função de viveiro dos estuários. Foram examinados a dependência e os movimentos de indivíduos entre duas áreas de viveiro distintas do estuário do Tejo, utilizando uma abordagem integradora baseada em assinaturas químicas em otólitos (Li:Ca, Mg:Ca, Mg:Ca, Cu:Ca, Sr:Ca, Ba:Ca, Pb:Ca) e assinaturas de isótopos estáveis em tecido muscular ( $\delta^{13}\text{C}$  e  $\delta^{15}\text{N}$ ). Ao longo do período do estudo, os resultados revelaram uma reduzida conectividade entre os dois habitats. A utilização simultânea destes marcadores naturais melhorou a avaliação da conectividade, reflectindo ligações ecológicas distintas, e forneceu informações complementares a escalas temporais distintas.

No capítulo 6, identificaram-se as áreas de viveiro de origem de indivíduos adultos de *P. flesus* e *D. labrax* recolhidos ao longo da costa Portuguesa, utilizando como base para a classificação as assinaturas químicas de otólitos previamente estabelecidas no capítulo 4. São poucos os estudos que até à data quantificaram a variação espaço-temporal da conectividade. Os resultados obtidos revelaram que as contribuições relativas das áreas de viveiro estuarinas para as populações costeiras de adultos variaram entre anos. Contudo, foi possível identificar alguns estuários que contribuíram de forma significativa para a reposição das populações costeiras nos dois anos, e outros cuja função de viveiro parece ser de menor relevância para estas espécies. Os resultados evidenciaram ainda a capacidade de dispersão destas espécies



de interesse comercial ao longo da costa portuguesa. Ao comparar as assinaturas químicas dos otólitos de adultos com a base de referência previamente caracterizada de assinaturas de juvenis, verificou-se a possibilidade de existência de áreas de viveiro alternativas que poderão ter contribuído para as populações de *P. flesus* e *D. labrax* ao longo da costa portuguesa. Em geral, considerando que a função de viveiro pode variar, com diferentes estuários a contribuírem desproporcionadamente para as populações costeiras, é fundamental ter um entendimento abrangente dos padrões de conectividade ao longo do tempo para múltiplas espécies chave, por forma a priorizar a protecção de habitats ecologicamente importantes, i.e. aqueles que de forma mais consistente promovem a manutenção e reforço das populações costeiras.

Por último, no capítulo 7, foi efectuada uma discussão geral que englobou as principais conclusões dos capítulos anteriores e onde são delineadas perspectivas futuras de investigação. Foram também tecidas considerações finais acerca da relevância do presente trabalho para o conhecimento geral sobre conectividade entre populações de peixes, sobre a sua importância e implicações para o desenvolvimento e implementação de medidas de gestão e conservação, bem como sobre a aplicação integrada de diferentes marcadores naturais.

## LIST OF PAPERS

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This thesis is comprised by the papers listed below, each corresponding to a chapter (Chapters 2 to 6). The author of this thesis is the first author in four papers and co-author with an equal contribution to that of the first author in the remaining paper. The author of the thesis was responsible for conception and design of the work, field surveys, sample collection and processing, laboratory analytical procedures, data analysis and manuscript writing of all the papers. Remaining authors collaborated in some or several of these procedures. All published papers were included with the publishers' agreement.

**CHAPTER 2:** River-Coast connectivity, estuarine nursery function and coastal fisheries

Rita P Vasconcelos, Patrick Reis-Santos, Henrique N Cabral, José L Costa, Maria J Costa

Published in Wolanski E, McLusky DS (eds) Treatise on Estuarine and Coastal Science, Vol 10. Academic Press, pp 81-107

doi:10.1016/B978-0-12-374711-2.01005-6

**CHAPTER 3:** Effects of temperature, salinity and water composition on otolith elemental incorporation of *Dicentrarchus labrax*

Patrick Reis-Santos, Susanne E Tanner, Travis S Elsdon, Henrique N Cabral, Bronwyn M Gillanders

*In Review* in Journal of Experimental Marine Biology and Ecology

**CHAPTER 4:** Temporal variability in estuarine fish otolith elemental fingerprints: Implications for connectivity assessments

Patrick Reis-Santos, Bronwyn M Gillanders, Susanne E Tanner, Rita P Vasconcelos, Travis S Elsdon, Henrique N. Cabral,

Published in Estuarine, Coastal and Shelf Science (2012) 112:216-224

doi:10.1016/J.ECSS.2012.07.02

**CHAPTER 5:** Connectivity within estuaries: an otolith chemistry and muscle stable isotope approach

Patrick Reis-Santos, Susanne E Tanner, Susana França, Rita P Vasconcelos, Bronwyn M Gillanders, Henrique N Cabral

*In review* in Marine Biology

**CHAPTER 6:** Connectivity between estuarine and coastal fish populations: contributions of estuaries are not consistent over time

Patrick Reis-Santos, Susanne E Tanner, Rita P Vasconcelos, Henrique N Cabral, Bronwyn M Gillanders

*In review in Marine Ecology Progress Series*

## **CHAPTER 1**

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**General introduction**  
**Aims and importance**  
**Thesis outline**

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## **General introduction**

### **Aims and importance**

### **Thesis outline**

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#### **General introduction**

Connectivity, or the exchange of individuals among geographically separated groups, is a critical component of marine fish populations as it drives population replenishment, determines colonization patterns and the resilience of populations to harvest (Thorrold et al. 2001, Cowen et al. 2007). Knowledge of this link is pivotal to understanding the dynamics, genetic structure and biogeography of many fish populations (Cowen et al. 2000). Moreover, knowledge of individuals' movement patterns underpins sustainable management and conservation efforts, the identification of ecologically important habitats and the design of protected areas (Cowen et al. 2006, Thorrold et al. 2007, Di Franco et al. 2012). At present, numerous marine and coastal fish species are commercially exploited and there is a growing consensus that they are under considerable and increasing stress from a plethora of human activities (Jackson et al. 2001, Vasconcelos et al. 2007a, Halpern et al. 2008, Trebilco et al. 2011).

Quantifying connectivity or the movement of marine organisms is a complex task. One of the main challenges relies on the ability to accurately track individuals over sufficient time periods to allow measurements of movement. Over the decades, this has been tackled by a variety of direct and indirect approaches (reviewed in Gillanders et al. 2003, Gillanders et al. 2011). Classically, movement was inferred via temporal and spatial abundance estimates coupled with size structure analysis (e.g. Blaber et al. 1989). However, only approaches via artificial and natural tags can provide reliable estimates of the rate and extent of movement. Despite the significant advances, amongst others, in the durability and miniaturization of artificial tagging techniques, they are still logistically difficult to execute in early life stages of fish, such as larvae and juveniles. This is largely due to small body size, high mortality and low recapture rates. Moreover, as most fish species are quite small at birth, and do not withstand the necessary handling until much older, movements between birth and age at tagging cannot be determined this way. These limitations have impelled the growing interest in the interpretation of distinct markers (e.g. chemical, genetic, parasitic) as natural tags to elucidate fish movement patterns and habitat use (Hobson 1999, Hellberg et al. 2002, Mackenzie 2002, Elsdon et al. 2008). The ability of these natural tags to unravel connectivity relies on differences in environmental conditions or genetic heterogeneity generating group specific tags, with the tremendous advantage that all individuals are foreseen to be tagged (Thorrold et al. 2002).

Amongst natural tags, the chemical composition of otoliths has become one of the most promising tools to quantify movement patterns as well as to reconstruct fish habitat use and

environmental life histories (Gillanders 2005, Elsdon et al. 2008, Walther et al. 2011, Sturrock et al. 2012). Otoliths are calcareous structures in the inner ear of teleost fish that aid in hearing and balance (Popper & Lu 2000), and present some specific properties that enable their use as archives of life history events as well as natural tags. Specifically, otoliths grow continuously by accretion of calcium carbonate (usually in the form of aragonite) forming daily and annual increments throughout fish life that can be used as chronological recorders of environmental conditions (Campana & Thorrold 2001); they are metabolically inert, once otolith material accretes its chemical composition is permanently retained (Campana & Neilson 1985); and finally, elemental incorporation is influenced by environmental conditions (e.g. water chemistry, temperature, salinity) and reflects, to some degree, the surrounding environment at time of accretion, albeit under some physiological or genetic control (Campana 1999). Hence, fish occupying areas with different environmental conditions are expected to present distinct otolith elemental signatures (also known as elemental fingerprints) and past environmental signatures can be assessed by analysing the portion of the otolith that corresponds to the life period of interest. In practice, otolith elemental composition has been used to determine stock identity and population structure (e.g. Thresher 1999, Campana 2005, Jónsdóttir et al. 2006, Tanner et al. 2012), reconstruct environmental life histories and migration patterns (e.g. Jessop et al. 2002, Limburg et al. 2011, Walther et al. 2011), discriminate natal or nursery origin (e.g. Gillanders & Kingsford 1996, Thorrold et al. 1998, Warner et al. 2005, Vasconcelos et al. 2007b, Wells et al. 2012) and to retrospectively determine the origin of adults (e.g. Thorrold et al. 2001, Gillanders 2002a, Rooker et al. 2008, Vasconcelos et al. 2008, Hamer et al. 2011). Nonetheless, the successful application of otolith chemistry to unravel connectivity and population structure depends on three assumptions being met: i) elemental tags must vary significantly between groups at spatial scales of interest; ii) all possible source groups are characterized; and iii) the signature should remain stable over the time period group assignments are to be made (Campana 1999, Gillanders et al. 2011). These assumptions are analogous to those required for other natural tagging approaches.

The stable isotope composition of soft tissues has also been successfully applied as a natural tag to examine movement and migrations in multiple systems and taxa (Fry 1983, Hobson 1999, Rubenstein & Hobson 2004, Herzka 2005, Trueman et al. 2012). Contrary to biomineralized tissues, isotopic composition of organic tissues is a result of synthesized and metabolised products mainly derived from diet. The approach is based on the premise that primary producer groups exhibit distinctive isotopic signatures that are propagated through a local food web. Use of soft tissue stable isotopic signatures for connectivity assessment requires an individual to experience a shift in prey with distinct isotopic composition following movement between habitats. This isotopic switch will gradually be reflected in the consumer's tissue, thus permitting transient and recent migrants to be distinguished from those fully equilibrated to the local food webs. Stable isotope approaches have tracked fish movement between discrete areas (e.g. Fry 1983, Rodgers & Wing 2008, Fuji et al. 2011, Dierking et al. 2012), and have proven to be particularly valuable to assess habitat use and small scale

movement patterns to, from and within estuaries (Fry 1983, Herzka 2005, Fry & Chumchal 2011, Green et al. 2012). Understanding small scale movement of individuals and differential habitat use patterns is crucial to determine habitat value and their ecological importance, particularly for predicting the potential consequences of habitat loss and environmental degradation at the level of ecosystem structure and functioning (Connolly et al. 2005).

Though the use of natural tags as tools to assess connectivity is a burgeoning research area, particularly concerning otolith chemistry, many core issues are still to be met regarding the spatio-temporal scales of connectivity, the underlying processes of dispersal and connectivity, the impacts of connectivity on population structure and dynamics, and effectively applying this information to management (Cowen & Sponaugle 2009). Moreover, as connectivity and population structure are influenced by behavioural and physical processes that act over a range of temporal scales, the simultaneous assessment of multiple natural tags on the same individuals should enhance our understanding of fish population connectivity by providing independent, but complementary, information at distinct spatio-temporal resolutions and hierarchical levels (Thorrold et al. 2002, Abaunza et al. 2008, Bradbury et al. 2008, Trueman et al. 2012).

Many marine fish species occupy spatially segregated habitats throughout their life history. In particular, estuarine systems are acknowledged worldwide for their nursery role for juveniles of numerous fish species (Beck et al. 2001, Gillanders et al. 2003, Able 2005). In these cases, adults generally live in the adjacent marine environment, whereas high densities of juveniles are found in estuarine nursery areas for months, or years, depending on the species, benefiting from enhanced conditions for increased growth and survival, until recruiting to the adult populations offshore (Miller et al. 1985, Koutsikopoulos et al. 1989, Beck et al. 2001). Estimating the successful movement of juveniles from putative nursery habitats and quantifying their effective contribution to coastal adult populations constitutes a mandatory component to correctly appraise nursery function (Beck et al. 2001). This is a vital link to understand connectivity and ascertain its influence on population structure and dynamics for these species. Ultimately, such information is essential to successful habitat conservation and fisheries management, prioritizing habitats and sites that effectively replenish adult populations, and delineating the appropriate spatial scales for management.

Along the Portuguese coast few studies have addressed coastal fish population structure using otolith elemental signatures (Correia et al. 2011, Silva et al. 2011, Correia et al. 2012, Tanner et al. 2012) or the connectivity between estuarine and coastal environments (Vasconcelos et al. 2008). The latter, Vasconcelos et al. (2008), provided the first evidence of connectivity between estuarine juvenile and coastal adult populations along the Portuguese coast. However, many aspects regarding spatial-temporal variability in otolith elemental signatures, changes in connectivity patterns over time, as well as numerous issues concerning the effective application of the otolith elemental composition to connectivity assessments and fish habitat use reconstructions remain unresolved.



### **Aims and importance**

The present study aims to assess the spatio-temporal variability in connectivity between estuarine and coastal systems, by means of otolith elemental signatures, and quantify the relative contributions of estuarine areas used by juveniles to coastal adult populations. Focus will be directed to estuaries along the Portuguese coast widely acknowledged for their nursery role to many commercially important species, namely flounder *Platichthys flesus* (Linnaeus 1758) and sea bass *Dicentrarchus labrax* (Linnaeus 1758) (e.g. Cabral & Costa 2001, Cabral et al. 2007, Martinho et al. 2008, Vasconcelos et al. 2010).

Otolith elemental composition has multiple applications and has been used in a wide variety of studies, yet many uncertainties still remain regarding the factors affecting elemental incorporation into otoliths (Elsdon et al. 2008, Thorrold & Swearer 2009, Sturrock et al. 2012). A laboratory approach was used to investigate the interactive effects of water chemistry, temperature and salinity on otolith elemental incorporation. Although discrimination of geographical origins of fish based on otolith chemistry does not require knowledge as to why otolith chemistry differs, the ability to reconstruct fish's environmental life histories relies on establishing predictable responses of otolith elemental incorporation to environmental variables, thus allowing past habitat use to be inferred via otolith chemistry.

A vital requirement to the successful application of otolith elemental fingerprints is the occurrence of spatial variation in otolith signatures at relevant scales. However, assessing temporal variation in elemental tags is also critical. It is likely that elemental signatures vary over time due to changes in environmental conditions. Such changes may confound spatial discrimination and have major implications for the retrospective identification of site or nursery of origin (Gillanders 2002b, Hamer et al. 2003) but are rarely considered. Therefore, a comprehensive evaluation of the spatio-temporal variation of estuarine elemental signatures is fulfilled. Established spatially explicit signatures will be the base for subsequent connectivity assessments between juvenile estuarine source sites and coastal populations.

Integrating natural markers, which provide information at distinct spatial and temporal resolutions, complements and enhances our knowledge on connectivity in multiple contexts. A combined approach of soft tissue stable isotopes and otolith chemistry was designed to assess the connectivity and habitat use between two segregated areas within an estuary. In addition, the relative contributions of individual estuaries to coastal populations are estimated over two distinct years. The latter provides valuable insights on spatio-temporal variations in connectivity and the extent of adult dispersal along the coast. Also of particular interest is the degree of population mixing in the marine environment. Estuaries may differ greatly in their nursery role, contributing disproportionately to adult populations (Gillanders 2005). If estuarine nurseries are to be effectively protected, knowledge on the generality of contribution patterns across multiple years is desirable and should contribute to a more judicious application of conservation resources. Both approaches outline much needed information to prioritize estuarine habitats for conservation, design protected areas, and allow sustainable fisheries management. Overall, a quantitative understanding of connectivity provides a valuable framework to evaluate the

benefits and potential effectiveness of distinct management and conservation strategies.

### **Thesis outline**

The present thesis is comprised of five scientific manuscripts published, or in review, in peer reviewed international scientific journals. Each corresponds to a chapter.

Estuaries are among the most productive and valuable ecosystems worldwide, yet they are also among the most threatened by human activities which jeopardize the long term viability of estuarine ecological functions and services. In this context, a comprehensive overview discussing the role of estuaries as nursery areas for marine fish is done in Chapter 2, followed by a critical review of the progress made towards the assessment of connectivity between estuaries and the adjacent marine environment. The main results and recent advances in the methodologies available to determine the contribution of estuaries to coastal populations are highlighted. Key findings are analysed in the context of evaluating the importance of connectivity measures and their significance to the development of management and conservation strategies and safeguarding coastal fisheries sustainability.

An experimental approach was used to assess in what way otolith elemental composition reflects water physicochemical properties (Chapter 3). Results are examined with the view to using otolith chemical composition as a proxy to reconstruct estuarine habitat use and fish environmental life histories.

In Chapter 4, the importance of temporal variability in the estuarine elemental signatures in juvenile fish is evaluated, and its potential role in confounding spatial discrimination among estuarine nurseries explored. Characterised otolith elemental signatures will be used as baseline data for the subsequent assessment of connectivity between juvenile and adult populations (Chapter 6).

The following two chapters (5 and 6) then investigate connectivity at different spatial scales, namely within an estuary (Chapter 5) and between estuaries and the open coast (Chapter 6). In Chapter 5, otolith chemistry and soft tissue stable isotope signatures were used to investigate connectivity and habitat use patterns within an estuary over time. The significance of integrating these distinct natural tags and their complementary use is discussed.

Otolith elemental signatures were used to determine the relative contributions of estuarine nursery areas to coastal fish populations in Chapter 6. Spatial and temporal patterns in connectivity are investigated and the most relevant nursery estuaries, namely those with higher contributions to adult populations, are highlighted.

In the final chapter, a general discussion addresses the main conclusions obtained from the different studies. The potential advantages of integrating distinct natural markers are also further appraised. Overall, a comprehensive evaluation of the findings presented herein and their implications towards habitat conservation and fisheries management is addressed. To finalise, future research perspectives are then discussed.

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## CHAPTER 2

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### **River-coast connectivity, estuarine nursery function and coastal fisheries**

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## River-coast connectivity, estuarine nursery function and coastal fisheries

**Abstract:** Due to their nature as transition systems, fishes in estuaries have affinities with both marine and freshwater faunas. Estuarine fish assemblages face physico-chemical dynamics and instability, as well as human induced changes. These systems represent a fundamental link in the life cycle of numerous species which use them in specific life stages (in obligatory or opportunistic manner). Many of these species represent important coastal fisheries and it is paramount to address the importance of estuaries as nurseries and their connectivity with the marine environment. Integrative approaches may provide tools towards sustainability and restoration of estuarine ecological functions.

**Key-words:** estuarine fish assemblages; coastal areas; freshwater flow; larvae; juvenile; nursery function; connectivity; coastal fisheries; management

### 1. Introduction

Estuaries are transition systems establishing the physical connection between freshwater and marine environments and are amongst the most biologically productive and valuable ecosystems in the world (Costanza et al. 1997). Simultaneously, they are among the most threatened by numerous human activities which damage and jeopardize the long term viability of their ecological functions (Vasconcelos et al. 2007). Agriculture, aquaculture, industry and engineering projects can alter the shape, nature and function of estuaries. Moreover, domestic and industrial discharges, including chemical contaminants and excess of nutrients, along with other pollution sources, and heavy fishing pressure are globally conspicuous and problematic (Haedrich 1983, Kennish 2002). Recent studies identify habitat loss and fragmentation as a greater problem than pollution itself whereas freshwater diversions are an emerging global issue (Cattrijsse et al. 2002, Kennish 2002). The introduction of exotic species is also a pressing issue (Galil et al. 2009). Such diverse anthropogenic activities represent numerous pressures and cause impacts on multiple ecological endpoints in estuarine ecosystems (Vasconcelos et al. 2007). Moreover, changes in estuaries due to human action may also imply consequences at a larger scale since estuaries and surrounding coastal areas are ecologically connected (Able 2005, Beck et al. 2001).

Despite their imperativeness, management, conservation and restoration measures aiming towards the sustainable development of a wide range of water and related resources have historically had limited and variable success. Even though integrated coastal zone management plans are drawn up worldwide most may be bound to fail in the presence of significant river input, because they commonly deal only with local, coastal issues, and do not take into account the entire river catchment as the fundamental planning unit (Wolanski et al. 2006). With this in consideration, the ecohydrology model approach, proposed by the UNESCO, stresses that the sustainable development of water resources is only possible if evolutionary established processes of water, nutrient circulation and energy flows are restored and maintained at the

basin scale (Chícharo & Chícharo 2006). It is increasingly recognized that a sound knowledge of ecosystem functioning is required as basis for the calibration of the relationship between hydrologic and ecological factors. The present chapter provides an overview of such relationship in what concerns fish communities. It addresses the structure of estuarine fish communities, their main patterns and trends in variability as well as influencing environmental factors. In addition, particular focus is given to the estuarine nursery function and the ecological link between estuaries and the adjacent marine environment, namely their importance to coastal/offshore fisheries. Finally, management and conservation strategies aiming towards the sustainable function of estuarine ecosystems and associated fish populations are also addressed.

## **2. Overview of estuarine fish assemblage structure**

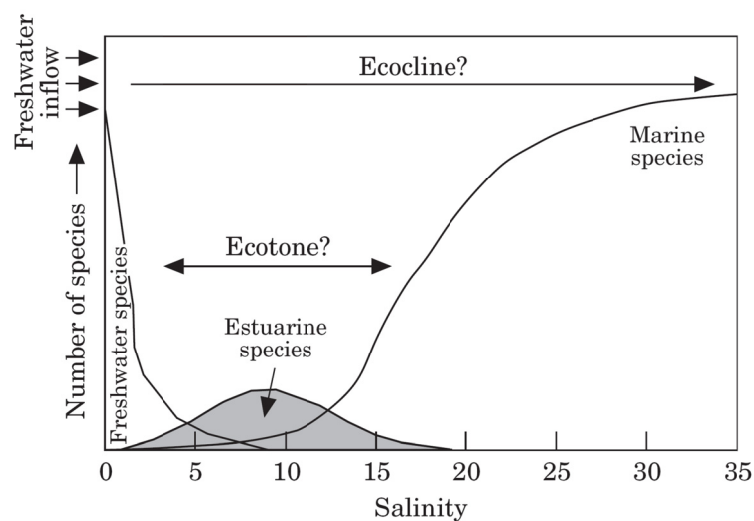
Fish species represent a diverse and abundant component of estuarine ecosystems, one which has received attention as a resource both for scientific research and commercial exploitation. In this section, we will focus on estuarine fish assemblages and present a brief overview of their composition, exploring worldwide diversity trends as well as influencing factors. The definition and use of ecological guilds is also summarized.

Estuarine fish assemblages have been studied worldwide however, the number of studies and available knowledge differs considerably according to geographical areas. Most studies have been conducted in European (particularly in Northern Europe), North American, South African and Australian estuaries (e.g. Whitfield 1998, Blaber 2000, Elliott & Hemingway 2002, Nagelkerken 2009). The present overview is a brief outline of available knowledge on estuarine fish assemblages focusing mainly on comparative and large scale analysis rather than an extensive review of studies on specific systems.

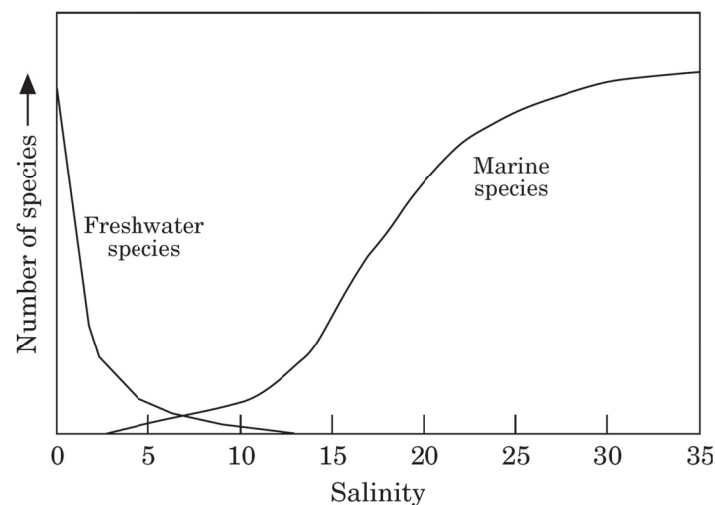
Due to their nature as transition systems, it is not surprising that fish fauna in estuaries has affinities with both marine and freshwater fauna as well as possessing a characteristic fauna of its own (Blaber et al. 1995). Nevertheless, there has long been an ecological debate regarding transition zones, such as estuaries, which can be seen as either ecotones or ecoclines (Attrill & Rundle 2002). Ecotones are areas of relatively rapid environmental change, which produce a narrow ecological zone between two different and relatively homogeneous community types. They are highly dynamic and usually unstable, resulting in an environmentally stochastic stress zone (tonus = stress or tension) (van der Maarel 1990). Earlier studies often describe estuaries as ecotones with a unique and highly adapted community between the marine and freshwater communities (e.g. Remane 1934, Reid 1961, McLusky 1983) (Figure 1).

Whilst analysing the macrozoobenthos communities from the freshwater Thames to the North Sea, Attrill & Rundle (2002) suggested the switch towards the ecocline definition, specifically defining estuaries as a two ecocline model. This is a more recent concept which refers to gradient zones containing relatively heterogeneous communities, more environmentally stable than those of ecotones. Overall an ecocline represents a boundary of progressive change (both spatial and ecological) between two systems and is a response to the

gradual difference in at least one major environmental factor (van der Maarel 1990). The Thames estuary closely fitted an ecocline model, with a continuum of assemblages along the salinity gradient from freshwater to the sea. It had two overlapping gradients defined by the major stressor (salinity/freshwater flow): from river to middle estuary for freshwater species and from sea to middle estuary for marine species. In the middle of the estuary there were freshwater or marine species at the limits of their range, rather than effective estuarine organisms (Attrill & Rundle 2002). This prompted the authors to redefine the Remane diagram by removing estuarine species in support of the arguments that there may be no true estuarine/brackish-water species (Barnes 1989) (Figure 2). Other studies have since sustained this view of estuaries as ecoclines (e.g. Greenwood 2007, Muylaert et al. 2009).



**Figure 1.** Remane diagram (after Remane 1934) annotated to show how the ecotone and ecocline models may apply in estuaries. The set of estuarine species may be an ecotone between marine and freshwater systems, or an ecocline could exist along the salinity gradient with a secondary factor being freshwater inflow (see text). From Attrill & Rundle (2002).



**Figure 2.** Remane diagram redrawn following the two-ecocline model, thus removing the estuarine species. From Attrill & Rundle (2002).

Despite the physical instability, the species composition of fish communities in estuaries along various biogeographic regions is relatively stable and fishes have more or less predictable patterns of abundance and distribution (Whitfield 1998). Such constancy in patterns can be attributed to: the occurrence and distribution of fish species, namely of specific ecological groups, along environmental gradients (e.g. salinity, temperature); the overall dominance of estuaries by relatively few species and the physiological constraints imposed on fish that use estuaries; seasonal movements in and out of estuaries; and the robust nature of food webs within estuaries (Moyle & Cech 1982, Kennish 1990, Whitfield 1998).

Estuarine ichthyofauna is in general abundant but characterized by the exceptional dominance of only a few species (Kennish 1990). In estuarine assemblages there may be more rare species than in fact the log normal model predicts, and Magurran & Henderson (2003) demonstrated how an ecological community can be separated into two distinct components. Persistent and abundant core species which are biologically associated with estuarine habitats present a log normal distribution; whilst occasional species, which occur infrequently and in abundance, have different habitat requirements and follow a log series distribution. These distributions are overlaid, producing the negative skew that characterizes real data sets.

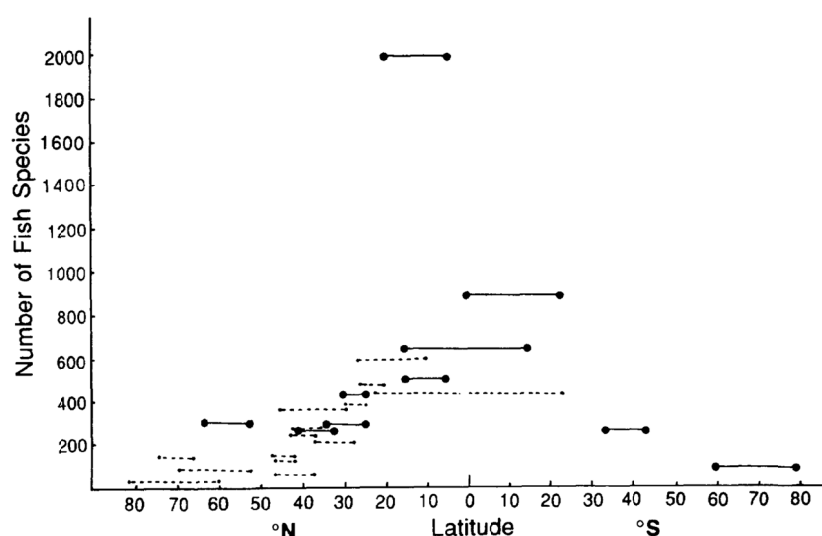
Estuaries are notably recognized for the high dynamics of their physico-chemical characteristics (such as salinity, temperature, water currents and dissolved oxygen) which fluctuate rapidly, both temporally (due to daily tidal cycles and seasonal variations) and spatially (Whitfield 1998). They are considered stressful for both marine and freshwater organisms as well as for those more adapted to the estuarine environment; although the latter are more tolerant to the changing environmental conditions and successfully capitalise on their use of the estuary. An advantageous cost-benefit relationship for temporary users was demonstrated by Yamashita et al. (2003) with higher physiological stress measured in juveniles within estuarine nursery grounds, via cortisol and growth rates, than in adjacent near shore areas.

According to Wallace et al. (1984) 100 to 150 species make regular use of the estuaries in southern Africa, though circa 1500 are found in the continental shelf. Even if direct comparison studies are scarce, overall fish species richness in estuaries tends to be lower than in the adjacent marine environment (Martino & Able 2003).

In an unpredictable environment (e.g. an estuary) only organisms that can tolerate wide perturbations are present and the ecosystem tends to be simpler yet more robust (Bruton 1989). In contrast, the more predictable marine environment may permit relatively more complex ecosystems, with increased species diversity, to evolve (Margalef 1968). Vrba (1980) suggested that the environment is the 'motor' of evolutionary change. Estuaries do not exhibit the environmental permanency necessary for eurytopes (generalists) to develop into stenotopes (specialists), and although some stenotopic species are found in estuarine systems, these taxa are forced to become survivors rather than increasers (Vrba 1980). For instance, adaptations developed by existing stenotopes in a particular estuarine system may be eliminated as a result of episodic (e.g. river flood cycle) or long term fluctuations (e.g. rise in sea level). The barriers to gene flow along estuarine and latitudinal gradients, together with intense natural selection

(Levinton 1980), have not resulted in widespread speciation contrarily to other more stable (predictable) aquatic ecosystems, such as the coral reefs of the Indo-Pacific or the great lakes of central Africa (Whitfield 1998).

A latitudinal gradient in coastal fish species diversity has been recognized in marine environments at a large scale, with an increase towards the equator. Greater species diversity in the tropics is concluded to be due to greater “effective” evolutionary time (evolutionary speed), probably as a result of shorter generation times, faster mutation rates and faster selection at greater temperatures (Rohde 1992) (Figure 3).



**Figure 3.** Number of coastal fish species at different latitudes. Large dots, unbroken line: Indo-Pacific; small dots, broken line: Atlantic. From Rohde (1992).

Similarly, a pattern of declining species diversity in estuaries with increase in latitude has been mentioned (Attrill 2002). The number of fish species in tropical and subtropical estuaries is usually an order of magnitude greater than in temperate regions (Blaber 2000). As a general trend, which allows for all exceptions, individual temperate estuaries may have 20-30 species (Potter et al. 1986, Elliott & Taylor 1989, Pomfret et al. 1991, Elliott & Dewailly 1995), warm temperate ones c. 50-60 (Darnell 1961, Lenanton & Hodgkin 1985) and most medium to large subtropical and tropical estuarine areas have at least 100, with some reaching over 200 (Blaber 2000). The higher diversity in tropical than temperate estuaries is yet to be thoroughly explained but it has been suggested to be due to the impact of successive glaciations on high latitude systems (Attrill et al. 2001). In the western Atlantic this decline was attributed to the increasing disturbance frequency or magnitude (e.g. temperature variations) with increasing latitude (Vieira & Musick 1993) and has been proposed to arise from the prolonged coexistence of species whose traits would otherwise lead to competitive exclusion.

In addition to this large latitudinal trend, a number of factors have been suggested to determine variation in fish species richness at a smaller latitudinal scale (e.g. at the level of

temperate European estuaries). The composition of estuarine fish assemblages depend upon the interplay of a whole range of factors, amongst which the most important are: estuary size, shape, depth and physical regimes, particularly salinity and turbidity, as well as habitat type availability; the nature and depth of adjacent marine waters and to a lesser extent, freshwater flow; and the geographical location of the estuary both in terms of latitude and in relation to marine features such as ocean currents, canyons and reefs (Blaber 2000).

In a comparison of 135 European estuaries (from Portugal to Scotland) Nicolas et al. (2010) concluded that system size was the main abiotic variable related to fish species richness (amongst variables mostly related to hydrology), which is in accordance with Wootton (1990). It is important to highlight that estuarine area may incorporate other aspects that corroborate the positive species-area relationship, namely the effect of sampling effort, as surveying larger areas implies a higher number of samples (Pihl et al. 2002), and may include higher diversity of habitats - higher number of ecological niches (Wootton 1990). Besides estuarine area, factors such as estuary mouth configuration, latitude and catchment area hydrology play a relevant role in determining fish species richness in estuaries. In addition to the effect of sampling effort and methodology, Pihl et al. (2002) suggested that habitat degradation throughout the individual systems, as well as the complexity of individual habitats and of adjoining marine and freshwater areas may mask possible differences related with habitat diversity.

There are many classifications of estuarine fishes based on distinct attributes such as salinity tolerance, feeding, reproductive or migratory habits. Nevertheless, these classifications are limited. For instance, salinity plays a specific role in the distribution of species within estuaries but overall a significant tolerance or degree of eurihalinity is a fundamental requirement for any species that inhabit them. More functional approaches have been developed based mainly on how fish use estuaries and where they spawn, their feeding preferences and strategies, and reproduction (Blaber 2000, Franco et al. 2008). As estuaries are transition environments inhabited by biota with highly distinct ecological characteristics, categorization of fish species into such ecological use functional groups, also known as ecological guilds or phenologies, has become increasingly popular (Elliott et al. 2007a). These approaches are useful in ecological studies as they enable the comparative analysis of communities disregarding the specific list of present species, as these necessarily vary between biogeographic regions, and focus on their ecological categories. Overall, guild approaches are a vital tool in understanding the functional and structural components of estuarine ecosystems, namely of estuarine fish assemblages, and may account for the changes in ecological niches due to the overall physico-chemical properties of estuaries (Elliott & Dewailly 1995, Franco et al. 2008).

In a review of estuarine fish assemblages by Franco et al. (2008), updating a previous review by Elliot & Dewailly (1995), numerous ecological guild classifications were re-assessed and summarized into the following groups: estuarine species, marine migrants, marine stragglers, freshwater species, anadromous species, catadromous species (Table 1). This classification will be used throughout the chapter when addressing ecological functional groups.

**Table 1.** Estuarine use functional group categories, their main characteristics and previous categorisations (synonyms) according to literature (original sources and later uses or modifications). Adapted from Franco et al. 2008

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**ESTUARINE SPECIES:**

May breed in the estuary; highly euryhaline species, able to move throughout the full length of the estuary

**Including**

Species spawning only in estuaries, where they complete their life cycle

Species with a marine larval dispersal phase, and postlarval stages and early juveniles returning to estuary to complete their life cycle

Species showing regular movements between the estuary and adjacent aquatic habitats

Species also represented by discrete marine populations

**Synonyms**

Truly estuarine resident; solely estuarine; estuarine & marine; estuarine residents; estuarine migrants; estuarine species; permanent residents; euryhaline freshwater species

**MARINE MIGRANTS:**

Spawn at sea and regularly enter estuaries in large numbers; highly euryhaline species, able to move throughout the full length of the estuary

**Including**

Marine species using estuaries as nursery grounds (entering as juveniles, staying there until the sub-adult stage is attained, then migrating seawards), either opportunistically or being dependent on estuarine nurseries (main representatives of the group)

Species spending much of their life within estuaries, either using them as nursery or visiting them regularly at adult stages

Species paying seasonal visits only at adult and sub-adult life stages

**Synonyms**

Marine seasonal migrant; marine juvenile migrant; marine estuarine opportunists; marine migrants; marine nursery; euryhaline marine species; marine estuarine-opportunists; marine estuarine-dependent

**MARINE STRAGGLERS:**

Spawn at sea; usually associated to coastal marine waters, enter estuaries accidentally in low numbers; predominantly stenohaline species, occur most frequently in the estuary lower reaches

**Synonyms**

Marine adventitious visitors; marine stragglers or adventitious visitors; marine stragglers; marine transient; marine species

**FRESHWATER SPECIES:**

Spawn in freshwaters

**Including**

Species entering regularly into estuaries, in moderate numbers, moving varying distances down estuaries

Species entering accidentally into estuaries, in low numbers, and restricted to low-salinity, upper reaches of estuaries and to periods of heavy freshwater flooding

**Synonyms**

Freshwater adventitious; freshwater; freshwater stragglers; freshwater migrants

**ANADROMOUS SPECIES:**

Live in the sea but regularly use estuaries as pathways of migration to freshwaters, where they reproduce

**Including** Species whose spawning run from the sea extends as far as the upper estuary, rather than going into freshwater

Species migrating from the sea to freshwater and in which the migration is not related to reproduction

Species (or a part of their populations) which may spend part of their life in estuaries

**Synonyms** Diadromous migrant; anadromous; semi-anadromous; diadromous; amphidromous

**CATADROMOUS SPECIES:**

Live in freshwater but regularly use estuaries as pathways of migration to the sea, where they reproduce

**Including** Species whose spawning run is only to estuarine areas rather than the marine environment

Species migrating to the sea from freshwater and in which the migration is not related to reproduction

Species (or a part of their populations) which may spend part of their life in estuaries

**Synonyms** Diadromous migrant; catadromous; obligate catadromous; diadromous; semi-catadromous; amphidromous

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Other ecological groups are also commonly considered in estuarine fish assemblage studies, such as feeding mode ecological groups or reproductive mode functional groups. These two classifications were also reviewed in Franco et al. 2008 (Table 2 and 3).

**Table 2.** Feeding mode functional group categories, their main characteristics and previous categorisations (synonyms) according to literature. Adapted from Franco et al. 2008.

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**MICROBENTHIVORES**

Feed mainly on benthic, epibenthic and hyperbenthic fauna, with prey size <1 cm

**Synonyms** Benthic invertebrate feeders; invertebrate feeders; bed feeders; hyperbenthos feeders; Benthophagous; hyperbenthophagous; meiofauna feeders; benthivores

**MACROBENTHIVORES**

Feed mainly on benthic, epibenthic and hyperbenthic fauna, with prey size >1 cm

**Synonyms** Benthic invertebrate feeders; invertebrate feeders; bed feeders; hyperbenthos feeders; Benthophagous; hyperbenthophagous; macrobenthos feeders; benthivores

**PLANKTIVORES**

Feed predominantly on zooplankton and occasionally on phytoplankton in the water column, mainly by filter feeding.

**Synonyms**

Zooplankton feeders; plankton feeders; zooplanktonic feeders; phytoplanktonic feeders; planktivorous; zooplanktivores; phytoplanktivores

**HYPERBENTHIVORES/ZOOPLANKTIVORES**

Feed just over the bottom, predominantly either on smaller mobile invertebrates living over the bottom and zooplankton; different feeding strategies for prey capture (ram, suction, or manipulation)

**Synonyms** Zooplankton feeders; benthic invertebrate feeders; carnivorous; zooplanktonic feeders; hyperbenthos feeders; planktivorous; hyperbenthophagous; plankton feeders

**HYPERBENTHIVORES /PISCIVORES**

Feed just over the bottom, predominantly either on larger mobile invertebrates living over the bottom and fish; different feeding strategies for prey capture (ram, suction; or manipulation)

**Synonyms** Fish feeders; hyperbenthos feeders; piscivorous; hyperbenthophagous; carnivorous; piscivorous; benthivores; piscivores

**DETRITIVORES**

Feed on all the small organisms in or on the surface layer of the substratum (e.g. benthic algae such as diatoms, microfauna including Foraminifera and Flagellata, and to a lesser extent smaller meiofauna) and associated organic matter (usually of plant origin); ingest relatively large volumes of sand or mud (by suction mechanisms), digest the food material and pass out the inorganic particles

**Synonyms** Detritivores; detritivores and scavengers; detritivorous; iliophagous

**HERBIVORES**

Graze predominantly on living macroalgal and macrophyte material

**Synonyms** Herbivores; herbivorous; grazers; browsers

**OMNIVORES**

Ingest both plant and animal material by feeding mainly on macrophytes, periphyton, epifauna and filamentous algae

**Synonyms** Omnivores; partly carnivorous; partly herbivorous; omnivorous; partial herbivores

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**Table 3.** Reproductive mode functional group categories, their main characteristics and previous categorisations (synonyms) according to literature. Adapted from Franco et al. 2008

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**VIVIPAROUS**

Internal fertilisation and live-bearing of young with a broad range of provisioning post-fertilisation, from no (strictly lecithotrophic viviparity, with live-bearing of young provisioned entirely by ovum yolk) to extensive provisioning beyond the nutrition provided by ovum yolk (matrotrophic viviparity)

**Synonyms** Viviparous, ovoviviparous; internal bearers

**OVIPAROUS**

Lecithotrophic maternal provisioning (limited to the yolking of ovarian oocytes prior to fertilisation) and external fertilisation; zygotes developing outside the maternal environment and independent of further energetic investment by either parent

**FURTHER DISTINGUISHED IN:**

**OVIPAROUS WITH PELAGIC EGGS**

Buoyant, pelagic eggs

**Synonyms** Oviparous; pelagic eggs; non-guarders; open substrate spawners; pelagic spawners

**OVIPAROUS WITH BENTHIC EGGS**

Demersal eggs settling on the substratum

**Synonyms** Oviparous; benthic eggs; non-guarders; open substrate spawners; benthic spawners

**OVIPAROUS WITH ADHESIVE EGGS**

Demersal eggs, adhesive and attached to substrata and/or vegetation

**Synonyms** Oviparous; adhesive eggs; non-guarders; open substrate spawners; benthic spawners, brood hiders

**OVIPAROUS GUARDERS**

Post-fertilisation parental care of eggs by guarding them externally, e.g. in a nest, where they develop into a post-larva or juvenile before being released into the surrounding waters

**Synonyms** Oviparous; guards; substrate choosers; nest spawners

**OVIPAROUS SHELTERERS**

Post-fertilisation parental care of eggs by sheltering them in a part of their body (e.g. mouth, brood pouch) where they develop into a post-larva or juvenile before being released into the surrounding waters

**Synonyms** Oviparous; sheltered eggs; external bearers

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According to Franco et al. (2008), fish diversity in temperate northern European estuarine assemblages is typically characterized by a majority of marine species, as also pointed out by Haedrich (1983) and Elliott & Dewailly (1995). A lower number of species was described to use these estuaries for spawning or permanent residence and even less species use these systems during diadromous migrations. In contrast, an important contribution or even dominance by species that complete their life cycles within estuarine systems is observed in many temperate western Australian and South African estuaries (Potter et al. 1990, James et al. 2007, Hoeksema et al. 2009). Many of these are closed to the ocean for substantial periods; therefore species that are able to spawn successfully within these systems are naturally favoured (Potter & Hyndes 1999). High contributions of estuarine species have also been reported in other European and tropical estuaries but this difference may be ascribed mainly to a different guild categorisation, namely in European studies, or to the difficulties in placing species into estuarine-dependent and non-dependent categories in tropical areas (Franco et al. 2008).

Besides representing the dominant proportion of estuarine fish assemblages, marine migrant species are the main focus of numerous studies worldwide as they are often also important economic fisheries resources in the coastal environment. This ecological group will be

addressed in further detail throughout this chapter in terms of their use of estuaries as nurseries, the connectivity between the estuarine and coastal populations and importance towards coastal fisheries.

### **3. Influence of freshwater flow in estuarine fish assemblages**

A central issue in estuarine ecology is the determination of which factors are relevant, and at what levels, in defining fish distribution patterns within estuaries. These are dynamic and often unstable environments and much of the observed temporal and spatial variability in physico-chemical and biological conditions is due to seasonal and annual changes in freshwater flow (Skreslet 1986, Sklar & Browder 1998). River flow plays such an increased role structuring abiotic and biotic factors in estuaries and coastal adjacent areas that its influence can be above that one of tides (Ande & Xisan 1989) whilst salinity, dissolved oxygen, nutrient concentration, stoichiometry, turbidity and productivity are all affected. Changes in river flow may be either of natural or anthropogenic origin and overall play a renowned role on the structure, functioning and composition of estuarine fish assemblages.

Trends in fish composition linked to river flow have been reported worldwide (e.g. Loneragan & Bunn 1999, Garcia et al. 2001, Kimmerer 2002, Whitfield 2005, Chícharo et al. 2006a, Martinho et al. 2007, Selleslagh & Amara 2008). In the present section an overview of the influence of river flow on estuarine fish assemblages is performed, and, within the scope of climate change, attention is given to events such as droughts and floods as well as to changes promoted by human use of water resources. To enable comparisons, focus is mainly directed towards the different ecological functional groups.

Potter et al. (1986) stated that estuarine fish communities change primarily due to extreme environmental episodes albeit many species and communities present clear temporal and spatial trends of occurrence related to diverse environmental factors within estuaries (Garcia et al. 2001, Cabral et al. 2007, Martinho et al. 2007).

The structure and functioning of estuarine fish assemblages, for instance in terms of ecological functional groups, is affected by changes in river flow. Nevertheless, few studies have quantitatively addressed these impacts. This is most probably due to lack of good datasets since comparable long time data series are required to enable the analysis of changes through time and the identification of specific patterns between environmental variables and species composition. Moreover, lack of comprehensive data may also lead to difficulties in isolating the factor or factors that effectively contribute to the observed changes.

Regarding marine migrant species, freshwater has been suggested as an important physical and chemical cue to facilitate and drive the orientation of migrating larvae and fish (Champalbert & Koutsikopoulos 1995, Whitfield 1999). The timing and magnitude of these increased flow events are important factors to enhance estuarine colonization by larvae and juveniles. For instance, species recruitment after periods of very high rainfall and flow in areas with large catchments may be faced with a physical barrier to recruitment (Loneragan & Bunn 1999). The use of particular sites and habitats as nurseries by juveniles may also be reduced or

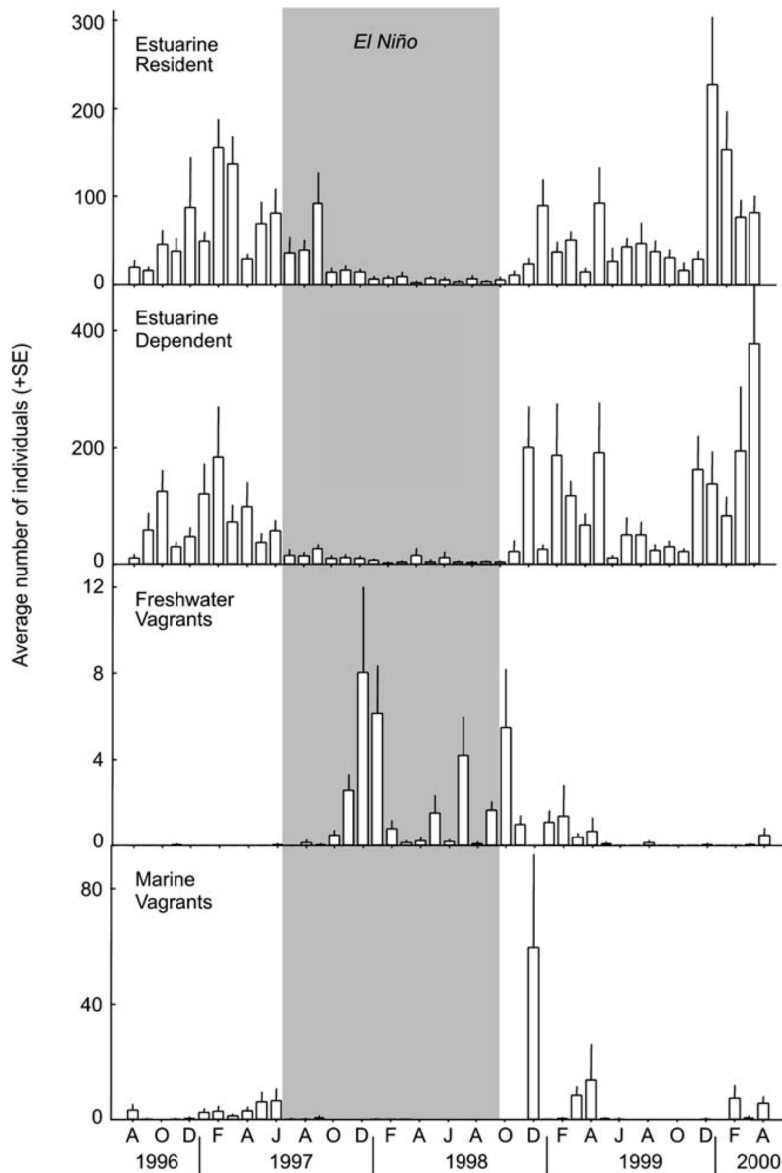
limited due to changes in salinity and turbidity caused by higher river flow (Loneragan & Bunn 1999). The duration of these events may tend to increase osmoregulatory stress, which fish may avoid due to their high mobility, and can lead to the emigration of these organisms from the estuaries.

The most pertinent impacts, regarding river flow, on estuarine resident species are related with reproductive success and changes to habitat availability. Larvae and juveniles of this ecological group should remain within the estuarine system (Whitfield 1999) and therefore these species present specific traits, such as benthic eggs (Pampoulie 2001), to minimize the risk of being flushed out. Increase in flow may transport these organisms to coastal areas possibly affecting their survival, although in specific cases it may lead to their occurrence in unusually high abundance (Garcia et al. 2001).

A growing concern on the influence of river flow on estuaries, namely regarding changes in fish assemblages, productivity and use of nursery areas is related to potential alterations brought by climate change and extreme weather events, such as droughts and floods. These alone can cause dramatic changes in river and estuarine systems (e.g. Garcia et al. 2001, Martinho et al. 2007).

Changes in the abundance of different ecological functional groups related with drought and flood events have been reported in several studies. In a temperate estuary of the Portuguese coast, a depletion of freshwater species and an increase in marine migrants was observed during drought years, as a result of an extension of saltwater intrusion in the estuary, as well as a reduction in abundance of estuarine species (Martinho et al. 2007). Nevertheless, in a comparison of wet and dry years in another estuary in the same coast Costa et al. (2007) observed increased densities of resident, marine migrants and catadromous species in the defined dry years and increased freshwater species in wet years. In El Niño periods, higher river discharge due to intense rainfall resulted in an increase in the number of freshwater fishes as well as in a temporary expansion of their distribution ranges throughout a large estuarine system in southern Brazil. During this period there was a decrease in the usually dominant euryhaline fishes (Garcia et al. 2003) (Figure 4).

Most southern African estuaries are closed off from the sea for varying periods (termed temporarily open/closed estuaries) by a sandbar which forms at the mouth. Mouth state is directly linked to freshwater input and its frequency, timing and duration plays a key role in determining species richness, composition, diversity and abundance. A decline in marine species number and abundance with increasing river flow was observed in a South-African temporarily open/closed estuary (Whitfield & Harrison 2003). Reduced river flow leads to longer mouth closures, and consequently less frequent or shorter openings, which hampers both immigration and emigration of marine fish species between an estuary and the coastal environment. Overall, permanently open estuaries have higher species richness but temporarily open/closed estuaries still provide important nursery areas for many species (Whitfield & Harrison 2003, James et al. 2007, Whitfield et al. 2008) including in other geographical areas (e.g. Gordo & Cabral 2001, Hoeksema et al. 2009).



**Figure 4.** Monthly mean fluctuations of the average number of individuals and standard error of the four fish groups (estuarine residents, estuarine dependents, freshwater vagrants, and marine vagrants) from August 1996 to April 2000. The 1997–1998 El Niño is delimited from July 1997 to September 1998. From Garcia et al. (2003).

Several studies have emphasized that freshwater input is essential to estuarine productivity since it is responsible for major nutrient inputs. Nonetheless, extreme river flood events may lead to reduced food availability for estuarine biota, due to the flush out of accumulated organic material which, if prolonged, may ultimately lower the fish carrying capacity of the system. In the particular case of smaller estuaries, these events can lead to very short water residency times, hindering both phyto- and zooplankton development (Wooldridge 1999, Whitfield & Harrison 2003).

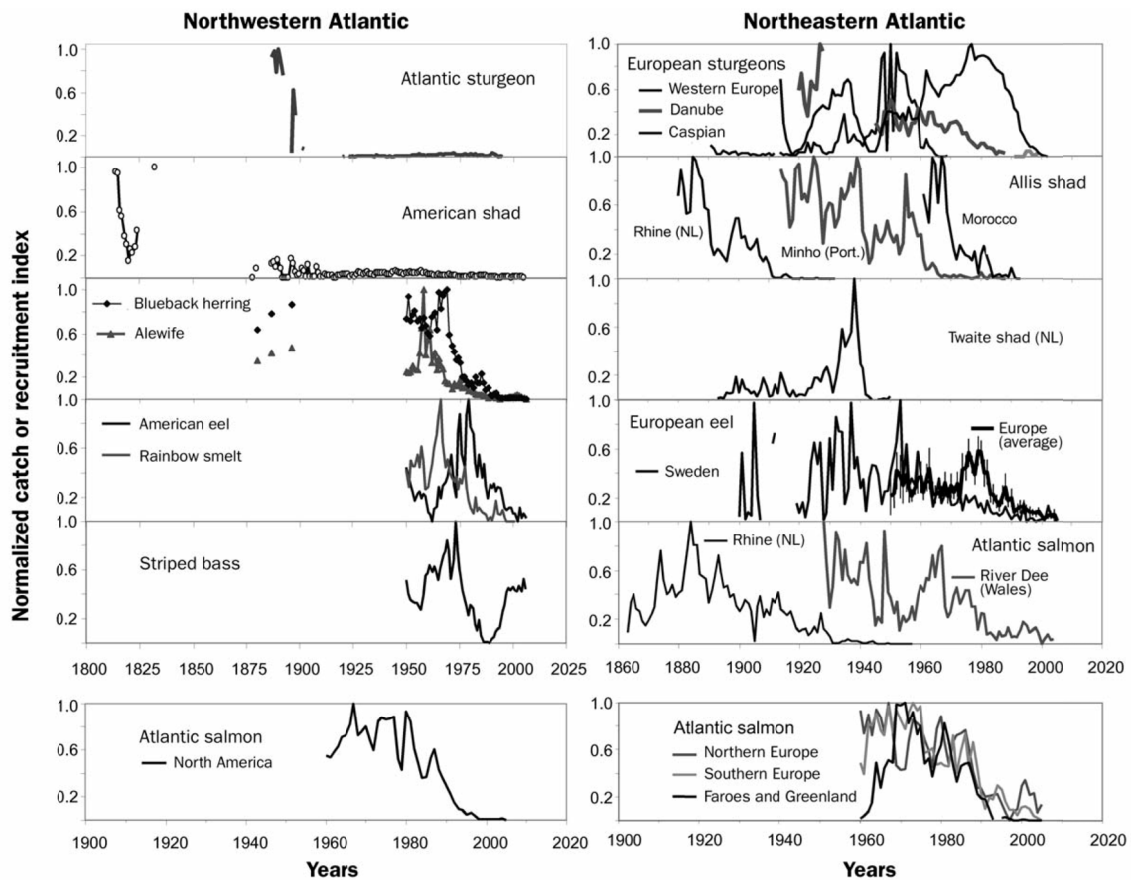
Flow effects on biological populations in estuaries have been proposed to be both positive and negative. Positive effects appear to operate through stimulation of primary production with the effects propagating up the food web (bottom-up regulation). Nevertheless, Kimmerer (2002)

suggested that the abundance of marine species in estuaries may respond negatively to flow simply as a response to physical habitat change (i.e. seaward displacement of their habitat) rather than as a result of upward trophic transfer, since in lower trophic levels a similar relationship was lacking. Accordingly, Fernández-Delgado et al. (2007) reported a reduction or enlargement of the estuarine area used as nursery grounds according to the variation of the isohaline position, as a result of the freshwater volume discharged from a dam during the previous week.

Following a drought period in an estuarine system in the Gulf of Mexico, there was a change in trophic diversity as well as a decrease in both biomass and species richness. In this system, river discharges control the food web structure and dynamics. Phytoplankton and benthic algal production responded rapidly to changes in discharges whilst higher trophic levels over a 1–2 years period (Livingston et al. 1997). In this same context of food web dynamics, the density of an estuarine-resident, a filter-feeding clupeid in a South African estuary, increased 15-fold during the months immediately following a flood, which could be indirectly linked to greater food resource availability in the form of phytoplankton and zooplankton blooms that followed the flood (Martin et al. 1992).

Changes in freshwater input, and consequently in salinity and seston, after the construction of a large dam, enabled marine species to colonize further upstream areas, which were previously dominated by freshwater species (Chícharo et al. 2006a). One of the most interesting features of this study was the observation of lower abundances of planktivorous and omnivorous fishes in the lower flow year, following the construction of the dam, whilst carnivorous fishes increased in the estuary. The authors state that fish are important regulators of estuarine trophic web processes and changes in dominant feeding groups can affect water quality, namely the occurrence of plankton blooms.

River flow is substantially altered for diverse human uses namely by regulation, retention or diversion. A large number of rivers and estuaries are affected by dams, levees, diversions, changes to watershed land use and bank reclamations, amongst others (e.g. Kennish 2002, Chícharo et al. 2006a, Vasconcelos et al. 2007). One obvious effect of building such barriers in river and estuarine systems is the blockage of migration routes for anadromous and catadromous fish species (Drinkwater & Frank 1994, Costa et al. 2001, Costa et al. 2008, Limburg & Waldman 2009). Accesses to historical spawning sites are cut causing population reductions and extirpations. Moreover, because many anadromous fish can use rivers as nurseries, the decrease in size and quality of marshes and other shallow habitats may affect recruitment. The relative abundance of 13 diadromous species in the North Atlantic has dropped to less than 98% of their historic levels and to less than 90% in other 11 species (Limburg & Waldman 2009) (Figure 5). Damming, habitat loss, over-fishing and pollution are the main pressures contributing to the drastic declines in numbers of these fishes and it may be hard to extract from these numbers the isolated, and probably lesser, effect of changes in river flow.



**Figure 5.** Normalized time series of indices of abundance of selected north Atlantic diadromous species. European eel includes standard errors of means for nine regions. The lower two panels compare Atlantic salmon. Unless otherwise stated, northwestern Atlantic data are US summary statistics. From Limburg & Waldman (2009).

Dams reduce the magnitude of downstream discharges, change seasonal flow patterns and reduce the frequency and magnitude of flood peaks (Loneragan & Bunn 1999). Maintaining daily freshwater pulses, through the release of freshwater from dams during critical periods, may be an important means towards avoiding cyanobacteria blooms. Understanding the relationships between the periodicity and magnitude of inflow pulse events, estuarine ecosystem structure and healthy functioning is a crucial step towards the development of management modeling tools (Chícharo et al. 2006b). Regarding artificial flow regulation, the maintenance of a seasonal pattern of flow coordinated with the timing of estuarine colonization by larvae and juveniles of marine migrant species may be more important than defining the magnitude of total annual flow (Loneragan & Bunn 1999).

It is important to notice that the construction of large dams also affects coastal communities (Aleem 1972, Almeida et al. 2004, Chícharo et al. 2006a). For instance, after the completion of the Aswan dam, nutrient concentration in coastal waters decreased markedly, catches of commercial planktivorous fishes declined c. 30 fold whilst prawns fisheries were reduced in half. This trend in lower catches of planktivorous fish is analogous to the one described by Chícharo

et al. (2006a). This link between estuaries and coastal fisheries is addressed in further detail in section 6.

#### **4. Estuarine nursery function and habitat use patterns**

A conspicuous feature of estuarine systems is the high densities of juvenile fish and crustacean decapods in many habitats, including species for which the adults live in adjacent marine areas. Estuaries are recognized worldwide for providing nursery grounds for juveniles of numerous fish species (Beck et al. 2001, Able 2005) and the present section focuses on the progress made on nursery function studies as well as on the main aspects addressed and methodologies applied.

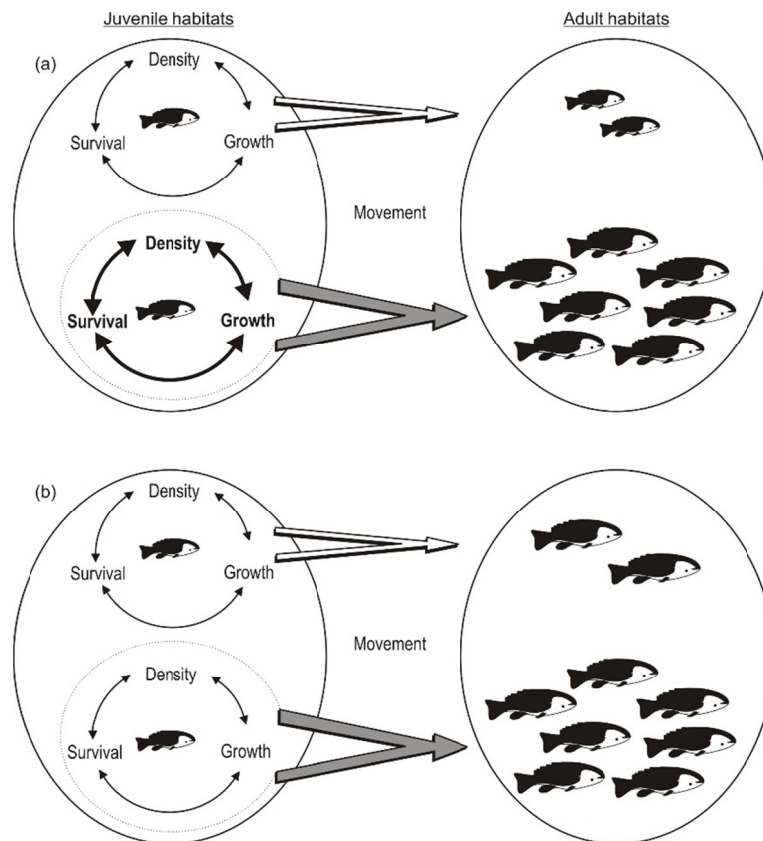
The nursery function of estuaries was recognized over a century ago for both invertebrates and fishes with complex life cycles (Hay 1905). The definitions and criteria for identification of nursery habitats have since evolved. A widely accepted view is that estuaries provide multiple benefits to juvenile fish, including abundance of food, shelter from large marine piscivores and favorable temperature regimes (Haedrich 1983, Miller et al. 1985). Moreover, the movement of fish larvae and juveniles from spawning areas to estuarine nursery areas could be considered from the perspective of costs and benefits (Miller et al. 1985): even though there is a physiological cost, overall juveniles of many species benefit from the use of estuaries (Yamashita et al. 2003, Le Pape et al. 2003a, Höök et al. 2008).

Whereas initially estuaries as a whole were considered to be nurseries, focus has been driven to particular areas within them or habitat types, such as salt marshes, mangroves, seagrass beds, oyster reefs or mud flats, where higher densities of juveniles are found (e.g. Orth et al. 1984, Costa & Bruxelas 1989, Adams et al. 2004).

The nursery concept has generally been applied *lato sensu* in numerous studies to areas where fish density, or growth or survival are enhanced (Désaunay et al. 1981, Costa & Bruxelas 1989, Koutsikopoulos et al. 1989, Gibson 1994). However, these approaches do not address whether habitats successfully transfer the higher juvenile biomass to adult populations (Beck et al. 2001). Moreover, not only have these studies been based for a long time on qualitative observations and putative assumptions, few efforts were made to define what constitutes a nursery, namely in a quantitative manner. A few years ago the 'nursery role' concept was redefined by Beck et al. (2001) and applied to sites or habitats which contribute per unit area to habitats used by adults with a number of juveniles above the average of others. The most important feature of this classification is that it provides a clear definition of nursery grounds as an objective and testable hypotheses for studies to be based on. The authors proposed that in these habitats or areas, ecological processes must support these greater contributions to adult recruitment from the result of the combination of four factors: (1) density, (2) growth, (3) survival of juveniles and (4) movement to the habitats used by adults. The successful combinations of these factors should maximize juvenile export and recruitment to habitats used by adults. More recently, Dahlgren et al. (2006) highlighted the relevance of 'effective juvenile habitats' (EJH) which are areas or habitats with a higher overall contribution to the habitats used by adults,



regardless of their unit area contribution. This approach is proposed to be more valuable in conserving habitats that provide highest overall contribution to adult populations but that would otherwise not be defined as nursery habitats following the definition by Beck et al. (2001) (Figure 6).

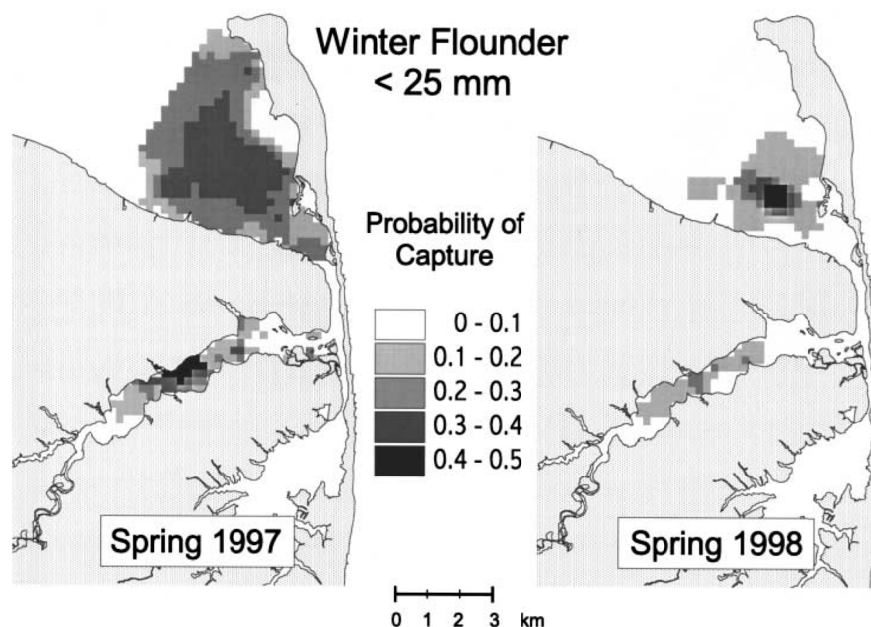


**Figure 6.** A representation of the factors operating in juvenile and nursery habitats. The thickness of the arrows indicates the relative contribution from each factor to the recruitment of adults. A nursery habitat (dashed oval) supports a greater than average combination of increased density, survival, and growth of juveniles and movement to adult habitats. (a) All four factors are greater in the nursery versus other juvenile habitats. (b) Only one of the four factors, in this case movement, is greater in the nursery versus other juvenile habitats. From Beck et al. (2001).

Quantifying successful movement of juveniles from putative nursery habitats to adults' habitats is therefore a mandatory component to correctly evaluate nursery function. Despite this, the relative value of juvenile grounds as nurseries is still often inferred from juvenile density and number (e.g. Le Pape et al. 2003b, Cabral et al. 2007, Dolbeth et al. 2008) and the dependence of adult stocks on these grounds addressed via the relationship between juvenile and adult growth, density or number amongst others (e.g. Rooper et al. 2004, see reviews by van der Veer et al. 1994, 2000).

The distribution of juveniles of marine migrant species within estuarine grounds and their use of particular areas or habitat types results from individuals' responses to multiple environmental variables, which can either be highly dynamic (e.g. salinity, water temperature,

food availability) or fairly stable (e.g. sediment type, presence of vegetation on the substrate) (Stoner et al. 2001, Selleslagh et al. 2009) (Figure 7).



**Figure 7.** *Pseudopleuronectes americanus*. Probability of capturing winter flounder <25 mm TL in the Navesink River/Sandy Hook Bay estuarine system calculated with the Generalized Additive Models for the size class and environmental variables (water temperature, sediment organic content, and salinity) observed in spring collections during 1997 and 1998. From Stoner et al. (2001).

Estuarine use patterns of marine migrant juvenile fish have been characterized for a vast number of species and estuaries however, most studies have conducted single estuary and single species approaches (e.g. Kelley 1988, Cabral & Costa 1999, Freitas et al. 2009). Such results represent only a part of the species overall scenarios of environmental use patterns and the degree to which they use different estuaries appears to vary both among and within estuaries (Able & Grothues 2007a, Cabral et al. 2007, Vasconcelos et al. 2010). Multi-estuary approaches provide enhanced evidence of species estuarine use and enable the direct comparison of arising patterns (Goldberg et al. 2002, Le Pape et al. 2003b, Cabral et al. 2007, Lazzari 2008, Selleslagh et al. 2009, Vasconcelos et al. 2010). In a comparison of nursery use patterns by five marine migrant species in nine estuaries, Vasconcelos et al. (2010) highlighted the intrinsically species-specific distribution patterns and the factors regulating variation within and amongst systems. Whereas salinity determined each species' main occurrence pattern within an estuary, species density is often influenced by other regulating factors (e.g. presence of vegetated substrate, sediment type and prey availability). Moreover, species occurrence and density amongst different estuaries is regulated by differences in environmental gradients within the distinct systems.

Small differences in growth and mortality rates in the first year of life are known to result in large differences in the number of individuals entering the reproductive stage annually

(Houde 1987, Cushing & Horwood 1994, Gibson 1994, van der Veer et al. 1994). It is generally agreed, upon that population size, namely for flatfish, is regulated at larval life stages and that carrying capacity of nursery areas is not reached (van der Veer et al. 2000).

Habitat quality in nursery grounds and its possible implications on connectivity are dependent both on the natural features of the estuary and also on the effects of anthropogenic factors that are conspicuous in estuaries worldwide (Able et al. 1999, Meng et al. 2001, Le Pape et al. 2007). High quality nursery habitats are assumed to be those where growth, survival and future reproductive potential are optimized (Gibson 1994). Even though habitat quality cannot be measured directly, it can be assessed based on individuals' responses.

Rapid growth rates are a commonly used indicator of habitat quality (Phelan et al. 2000, Ross 2003) as they imply that: there is sufficient food availability; individuals will be larger by the end of the Summer growing season; and achieve size refuge from predators increasing their chances of over-Winter survival (Sogard 1997). The classical approaches to study growth rates consist in modal progression analysis of length frequency distributions, using methods as the popular Bhattacharya (e.g. Cabral 2003). Analysing growth strictly in length is mainly adequate for small fish but less suitable for larger ones since at a certain point fish increase faster in terms of biomass than length.

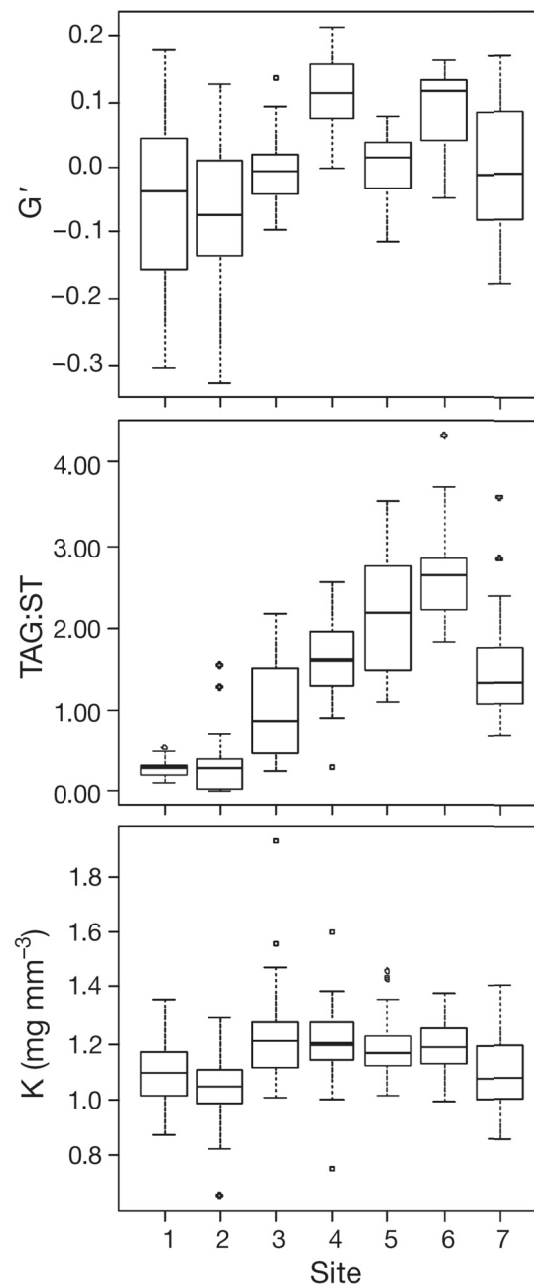
The use of direct measures of growth in nursery studies implies tag-recapture (Able et al. 2005) or in-situ caging methodologies (Able et al. 1999, Sogard 1992, Kuropat et al. 2002, Meng et al. 2001). However, these techniques are often avoided due to difficulties in obtaining results. Moreover, caging may affect survival and initially reduce feeding and growth rates due to physiological stress (Oikari 2006). For these reasons several alternative and indirect methods have been developed.

Condition indices are efficient proxies of growth rate and nutritional status and provide information on fishes' responses to habitat quality which, in estuarine nurseries, can be indicative of differences in the nursery role of estuaries or habitats within them (Suthers 1998, Buckley et al. 1999, Fukuda et al. 2001, Amara et al. 2007) (Figure 8).

Nucleic acid analysis is an acknowledged practical tool to study recent overall nutritional condition and growth of larvae and young fish, as well as their responses to environmental variability (Buckley et al. 1999). Specifically, RNA:DNA ratio reflects variations in protein synthesis rates: RNA concentration increases with food availability and protein requirement, while DNA somatic content remains relatively constant. RNA:DNA ratio has been shown to respond to changes in feeding conditions and growth in periods as short as one to three days in a variety of fish species, and is a valid and reliable growth rate estimator applied in numerous field assessments (Rooker & Holt 1996, Buckley et al. 1999, Fukuda et al. 2001, Gwak & Tanaka 2001, Mercaldo-Allen et al. 2006, Vasconcelos et al. 2009).

Lipid storage indices may be used to evaluate nutritional status (Amara et al. 2007). For instance, triacylglycerols to sterols ratio (TAG:ST) reflects the quantity of TAG reserve lipids to the quantity of ST structural lipids in fish. The TAG content is dependent on the nutritional state of fish, as TAG are a principal reserve of energy in teleosts and the first components to be

mobilised during periods of stress, while sterol contents remain essentially unchanged during starvation (Galois et al. 1990).



**Figure 8.** *Solea solea*. Mean growth index ( $G'$ ), triacylglycerols:sterols ratio (TAG:ST) and Fulton's condition index ( $K$ ) for 0-group sole caught in 7 nursery grounds in the French coast. The boxes represent the interquartile range containing 50% of the values. The whiskers extend to the highest and lowest values, excluding outliers. The line across the box indicates the median. From Amara et al. (2007).

Otolith microstructure analysis, namely the identification of daily increments has allowed the determination of fish age (in days) and therefore estimates of growth rates during the juvenile period, in combination with fish length (Amara et al. 2007).

Laboratory studies have developed growth models for juveniles of numerous species, mostly identifying temperature, food availability and salinity as determinant factors (see review by Buckley et al. 1999). Large differences in juvenile growth rates in estuaries have been

reported at vast latitudinal scales (Cabral & Costa 2001, Vinagre et al. 2008a) as a result of the influence of temperature on growth (Fonds 1975) since it governs the rate of chemical reactions, pacing metabolic requirements, digestion and the rate of growth (Fry 1971). At smaller scales, i.e. amongst closely located estuaries or sites within the same estuary, differences in growth have been widely reported as a result of several environmental factors, both biotic and abiotic (Kuropat et al. 2002, Gilliers et al. 2006, Vasconcelos et al. 2009). Growth rates have also been related to differences in site prey availability (Islam & Tanaka 2005) and observed correlations between juvenile growth and density (Vasconcelos et al. 2009) may also be related with prey availability as higher competition for resources potentially occurs at higher densities. Regarding the importance of density dependent processes in nursery grounds, growth has been found to be limited by high juvenile densities whereas in other cases no relationship was found (Vasconcelos et al. 2009). On the other hand, growth indices measured in estuaries along the Atlantic coast of France correlate with anthropogenic disturbances (Amara et al. 2007) as depressed physiological condition and growth occurs in the presence of numerous xenobiotics (van der Oost et al. 2003, Fonseca et al. 2009).

The most direct approaches to assess survival are based on artificial tagging and mark-recapture methods. A mark-recapture study in coral reef systems, via the tagging of specimens immediately after settlement and the recapture of survivors 30 days later whilst simultaneously analyzing otolith growth, identified features associated with enhanced survival probability, namely that survivors exhibited relatively slow initial growth during their first few days on the reef, followed by a period of accelerated growth (Gagliano & McCormick 2007). Through a different approach, comparing species characteristics in surviving early juvenile stages on the reef with those of the cohort from which they originated, planktonic growth history was identified as the most influential and long-lasting trait associated with juvenile persistence (Gagliano et al. 2007). It is important to point out that one of the confounding aspects in quantifying survival is accounting for potential movements of fish which may influence natural mortality estimates. To avoid this misclassification, some studies on survival use the term persistence instead.

In contrast to coral reef environments, less attention has been given to survival in estuarine environments and it is difficult to directly extrapolate results. Juvenile survival in estuaries has been related to abiotic variables, such as temperature, dissolved oxygen, salinity and pH using caging and tagging methods (Necaise et al. 2005), although no differences in growth were reported. In laboratory experiments, Shervette et al. (2007) reported rapid growth and high survival at typical estuarine salinities (15-30). Mortality rate estimates, from declines in abundance-at-age plots, indicated substantial mortality during the early post-settlement period in estuaries (Rooker et al. 1999) and in areas where growth rates were equivalent improved survival likely optimizes fitness (Ross 2003). Although juveniles of numerous fish species can occupy both estuaries and their adjacent shallow coastal areas, estuarine nurseries have been shown to be associated with higher densities, growth rates and condition of juvenile fish, in spite of the associated physiological cost (Le Pape et al. 2003a, Yamashita et al. 2003, Höök et al. 2008).

The importance as nurseries of the different habitat types found in estuaries for marine migrant species has been investigated in a wide number of studies. However, there are only a few integrative approaches which have provided more comprehensive evidence on estuarine habitat as nurseries in the context of Beck et al. (2001). In a review by Minello et al. (2003) the importance of saltmarshes as nurseries for nekton (comparatively to seagrass, open water, macroalgae and oyster reefs) was assessed based on growth, density and survival. Analysing fish density, habitat types were ranked from highest to lowest as: seagrass > vegetated marsh edge, non-vegetated marsh, open water, macroalgae, oyster reefs > vegetated inner marsh. On the other hand, growth was similar in saltmarsh, open water and macroalgae beds and lower in seagrass. In comparison to saltmarshes, survival in oyster reefs was higher but lower in open water whilst it was similar to the one in seagrass. Overall, nursery value was higher in seagrass, followed by saltmarsh and open water, whilst vegetated marsh appears to have a higher nursery value than non-vegetated marsh. Similarly, Nagelkerken (2009) reviewed the nursery role of mangrove and seagrass habitats in tropical systems and through the comparison of several studies concluded that mangrove and/or seagrass habitats generally presented higher fish densities or showed higher survival than coral reefs, whilst growth rates were never highest in mangroves or seagrass beds. Nevertheless, these integrated approaches do not evidence the movement from these habitat types and are therefore still not sufficient to evaluate their effective nursery value and determine if in general these habitats act as nurseries or EJM for fish, as also discussed in both reviews (Minello et al. 2003, Nagelkerken 2009).

As this brief overview pointed out, several studies have investigated the potential of estuaries and their habitats as nurseries. Nevertheless, great advancements in this research area came with the development of several techniques which have enabled the determination and quantification of fish movement. Their role in determining the connectivity between estuaries and the marine environment as well as to identify nursery areas is addressed in the next section.

## **5. Links between estuarine nurseries and coastal stocks**

The exchange of individuals amongst geographically separated groups, or connectivity, is a critical property of marine populations (Cowen et al. 2000). This is particularly important for fish species with complex life cycles, such as diadromous or marine migrants using estuaries or coastal areas as nursery grounds, which perform migrations associated with different stages of their life cycle. Assessing connectivity of marine migrant fish populations is essential to establish their spatio-temporal dynamics and the links between larval dispersal and supply, juvenile abundance, survival and contribution to adult stocks as well as to identify important areas and habitats (e.g. nurseries). Understanding the links between estuarine and coastal marine environments is a complex challenge but it is a vital asset to identify ecologically important habitats. Throughout this section we will discuss the advancements of connectivity studies highlighting the main results and applied methodologies in determining the contribution of estuaries to coastal populations.

Adults of marine migrant species inhabit the marine coastal environment and after spawning post-larvae or early juveniles are transported to coastal areas and estuaries. Individuals remain in these areas (for months or years depending on the species) where they grow to sub-adult stages and then recruit to habitats used by adults, located off-shore (Miller et al. 1985, Koutsikopoulos et al. 1989). As these species' eggs and larvae are pelagic, their transport to coastal areas and estuaries and their distribution and settling sites will partly depend on water mass transport (Nielsen et al. 1998). Long larval periods and wide larval dispersal represent ecological strategies through which connectivity among populations is potentially strengthened, new settlement habitats may be colonized, gene flow enhanced and intra-specific competition minimized (see DiBacco et al. 2006). However, the extent to which this occurs remains uncertain since the links among larval sources and sinks, dispersal potential, realized transport and successful recruitment are rarely quantified (Swearer et al. 2002); yet up to date they have been best characterized for reef fishes (Jones et al. 1999, Swearer et al. 1999, Thorrold et al. 2001, Almany et al. 2007). One of the main issues currently in discussion is the extent to which fish populations are open or closed (Cowen et al. 2000). The initial belief was that most were demographically open but recent evidence contradicts this concept of broad dispersal and highlights the importance of local replenishment (Swearer et al. 2002, Almany et al. 2007). This change of paradigm is one of many indications that there are still a number of core challenges that have to be overcome before routine estimates of connectivity are possible, namely determining the spatio-temporal scales of connectivity, the underlying processes of dispersal and connectivity, as well as the impacts of connectivity on population structure and dynamics (Cowen & Sponaugle 2009).

Along-shore wind stress and density differences, i.e. buoyancy-driven flow, have been identified as key factors in larvae transport along the continental shelf for several species and areas. Vertical migration by larvae has been shown to have strong effects on transport since parcels in the water column may move in different directions or at different speed. Vertical migration in and out of surface waters may influence the likelihood of onshore transport by wind currents, internal waves or tidal bores (reviewed in Shanks 1995). Moreover, many of these fish species use, at some part of their larval cycle, selective tidal stream transport from the continental shelf to estuarine nurseries (Jennings & Pawson 1992, Jager 1999, Amara et al. 2000, van der Veer et al. 2009). Selective tidal stream transport is accomplished when larvae migrate up in the water column during flooding tidal currents and downward during ebbing tidal currents (Epifanio 1988). This allows larvae to overcome the seaward flow of the estuary and be retained in estuarine nursery areas (e.g. Boehlert & Mundy 1988). Even though stimuli for these vertical migrations are yet to be clarified, the role of salinity, pressure, micro-turbulence and food has been investigated (see Rowe & Epifanio 1994).

In summary, larval fish settlement in estuarine nursery areas is the end result of numerous biological and physical processes, with larval supply and available settlement habitat as the main factors promoting differences in the densities of newly settled individuals.

While the dynamics of recruitment have been of interest to marine ecologists for some time,

the ability to effectively identify natal or nursery origin of recruits is relatively new (see DiBacco et al. 2006). This is mostly due to the technological development of several methods that have enabled the identification and quantification of fish movement (Gillanders et al. 2003).

Tagging has been widely used to determine movements of fish. Tagging methods can be divided into five broad groups including external tags (trans-body tags, dart-style tags and internal anchor tags), external marks (fin clipping, branding and pigment marking), internal tags (coded wire tags, passive integrated transponder and visible implant tags), telemetry (acoustic telemetry and archival tags) and natural marks (morphometric marks, meristic marks, parasitic marks, chemical marks and genetic marking).

Although in constant development, particularly due to the advances in miniaturisation of artificial tags and telemetry, most techniques are not usually viable in small juveniles or larvae (see reviews by Gillanders et al. 2003, Pittman & McAlpine 2003). Nevertheless, artificial tagging techniques have been extensively used to assess movements of juveniles within an estuary, their habitat use and site fidelity (see Saucerman & Deegan 1991, Potthoff & Allen 2003, Able et al. 2005). Moreover, some studies have successfully used artificial tags in juveniles to address connectivity between estuaries and coastal areas (see review by Able & Grothues 2007b). For instance, external marks and mark-recapture methods have been applied to determine the home range and residency of juveniles of two sparids in a coastal lagoon system and reported a high residency index between first and last detections, whilst some sub-adults, with acoustic tags, were found to leave the system to the adjacent coastal area (Abecassis et al. 2009). Also using acoustic tags, Fairchild et al. (2009) analyzed movement and dispersal patterns of juvenile fish in an estuary and registered that approximately 20 % of tagged individuals left the estuary mainly in winter whilst 50 % remained in the estuary.

The use of parasites as natural tags of habitat use is based on the presence of parasites in a group of fish and their absence in others, or at least different infection rates or genetics. One major difficulty in their use in assessing estuarine nursery connectivity is identifying parasites that spatially discriminate nursery areas but also remain within the host after movement from juvenile to adult environments, whilst the latter are not susceptible to further infection (MacKenzie & Abaunza 1998). In a recent study, parasite infections in freshwater juveniles of a catadromous mullet highlighted their movement between freshwater and marine environment and evidenced the importance of local contribution to fisheries (Lester et al. 2009). Although seldom used in the context of marine migrant species, parasitic infection rates have enabled the identification of an estuary as a nursery ground for a flatfish (Olson & Pratt 1973), and differential parasite infection and genetics have widely been used to determine stock composition (Oliva & Ballon 2002, Abaunza et al. 2008).

Population genetic structure is a consequence of gene flow, drift and selection, and is a valid tool to analyse connectivity. Genetic methods can be used to estimate migration rates of individuals between and among populations although in most cases their use is best addressed on a longer time scale rather than on a single generation as the remaining markers (Hedgecock et al. 2007). Genetic methods are unique in measuring effective dispersal between populations

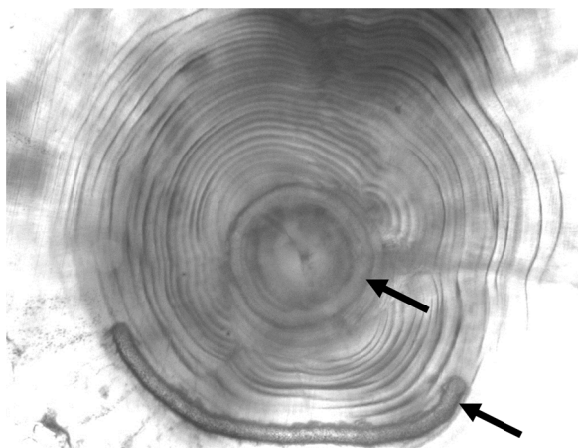


since they can determine those individuals that survive and breed after dispersal to a new population (Purcell et al. 2006). Microsatellite markers have been used to estimate connectivity among populations: through indirect estimates of migration based on the differentiation between populations; through direct estimates of migration based on assigning individuals to source populations; and using genes as natural tags (Carmen & Ablan 2006). Nevertheless, only a few studies have applied them to explore the link between estuarine and marine environments; for instance, to determine if anadromous fish populations are structured at the scale of individual estuaries, in accordance with the member vagrant hypotheses (Bradbury et al. 2008). Variations in mitochondrial DNA demonstrated an isolation-by-distance model in several marine migrant species in the Gulf of Mexico and eastern coast of the U.S.A. (Gold & Richardson 1998). This indicated that fish from neighbouring estuaries and bays were more genetically similar to one another than to those from more distant systems. Among estuarine nurseries of the Bay of Biscay there was no genetic differentiation for age-0 and age-1 fish, but there was among sub-adults. These populations appear to be spatially structured between a southern and a northern group exhibiting different genotypic distributions, essentially as a result of impact by selection on metallothionein locus in southern estuaries. This is one of the most interesting aspects of this research as southern nurseries are affected by cadmium, indicating a possible correlation between observed genotypic distributions and nursery habitat features (Guinand et al. 2008).

Recent studies with natural tags consisting of the chemical composition of calcified structures show great promise in revealing the movement of individual fish and connectivity patterns of fish populations (Elsdon et al. 2008). The chemical composition of fish otoliths is one of the most valuable natural tags of habitat use and this is due to some specific otolith properties. They are metabolically inert, grow continuously and the chemical elements of the organism's surrounding environment are accreted onto the growing surface of the otolith and permanently retained (Campana 1999, Thresher 1999, Gillanders 2005, Elsdon et al. 2008). The environmental history of a fish at a specific time period in the past can thus be determined by analyzing the chemical composition of the portion of otolith that corresponds to that specific time period (Brown 2006). To assess habitat use with natural tags, these should be consistent over time and all possible source habitats characterized (Campana 1999).

Analysis of major and minor element composition of otoliths, as well as  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  stable isotope ratios, has been used as a tool for the identification of: (1) individuals that have different migratory patterns (Kalish 1990, Tsukamoto & Arai 2001), (2) populations or stocks of fish (Campana 1999, Rooker et al. 2003) and (3) natal origin or nursery origin to near shore and estuarine habitats (Gillanders & Kingsford 1996, 2000, Thorrold et al. 1998, 2001, Dorval et al. 2005, Brown 2006, Rooker et al. 2008). The rationale for the latter objective is that coastal fish that spent their juvenile period in an estuary are expected to have an otolith elemental fingerprint which reflects their estuarine life period (Figure 9). Exciting results include the retrospective assignment of adult fish, captured in marine environment, to: their source nursery habitat type (e.g. Gillanders & Kingsford 1996, Forrester & Swearer 2002, Brown 2006, Fodrie & Levin 2008); individual or groups of estuaries (e.g. Gillanders 2002, Vasconcelos et al. 2008); or

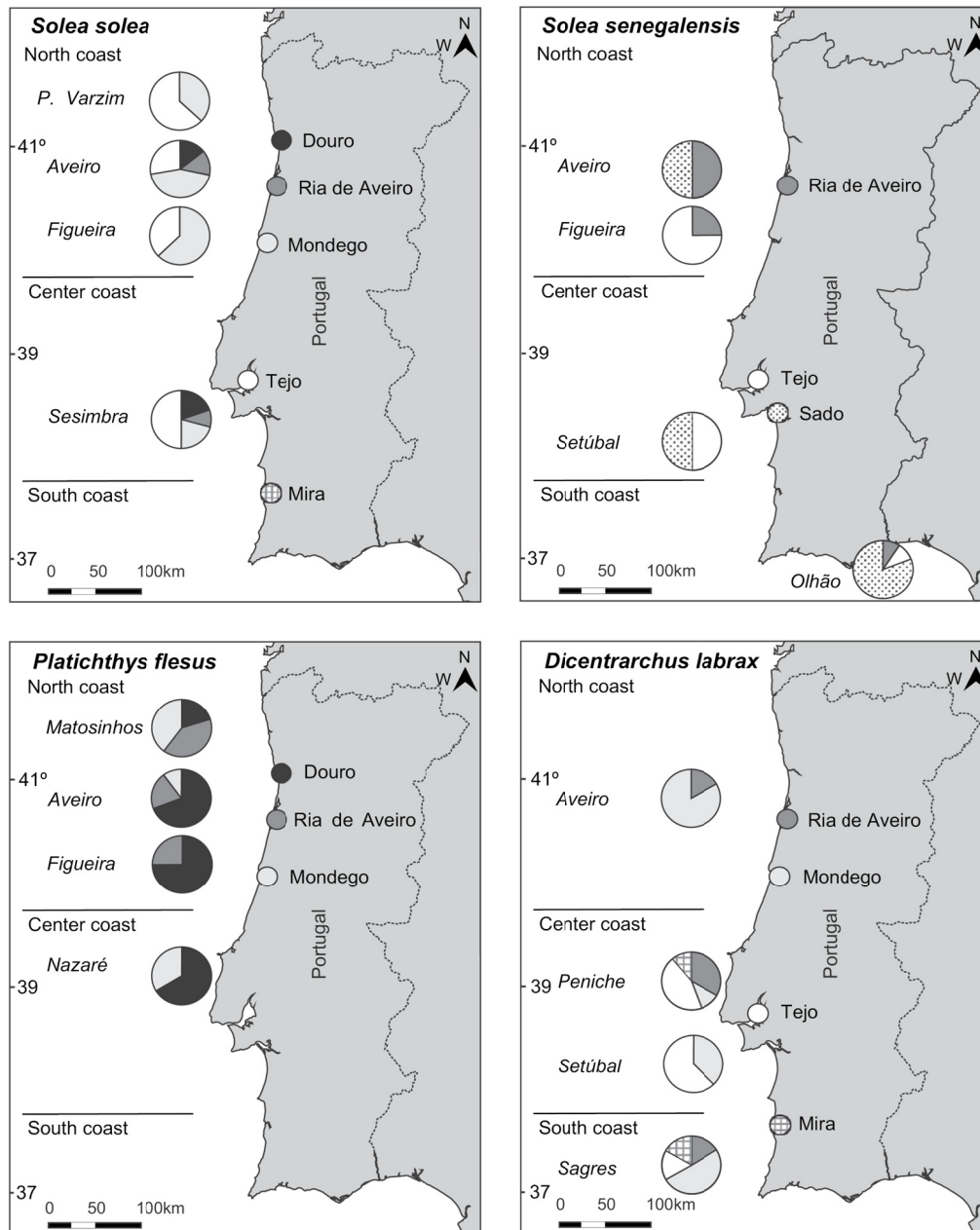
sites within estuaries (Chittaro et al. 2009) (Figure 10). Overall, these provided information on the relative contribution of different nursery areas or habitats.



**Figure 9.** *Solea solea* otoliths showing concentric daily increment marks. Arrows indicate two ablated areas, one in the post-larval growth area, after the metamorphosis mark, and one in the juvenile growth area. Chemical composition of the otolith was analysed with Laser Ablation - Inductively Coupled Plasma Mass Spectrometry. Author: S. Tanner.

The use of soft tissue stable isotope composition ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$ ) is also of great use in connectivity studies, namely to assess movement to, from and within estuaries due to the diverse signatures found among the assorted habitats. Since specific primary producer groups tend to exhibit distinct isotopic signatures, which are reflected along the food web, the movement of organisms to a new habitat causes a shift in isotopic composition which can be used as an indicator of site fidelity or connectivity. Changes in tissue isotopic composition are gradual and it is therefore possible to distinguish recent immigrants from organisms that have been in the habitat for a longer period (Fry 1983, Herzka et al. 2002, Herzka 2005). Stable isotopes have also been used to assess site fidelity and mixing among subpopulations within estuaries (Vinagre et al. 2008b, Weinstein et al. 2000). This is of particular interest since marine migrant species may, for example, simultaneously use distinct sites and habitats within a system before recruiting to coastal stocks. In comparison to the use of other natural marks within estuarine systems, e.g. otolith chemical composition, stable isotopes may provide information at a smaller spatial and temporal resolution.

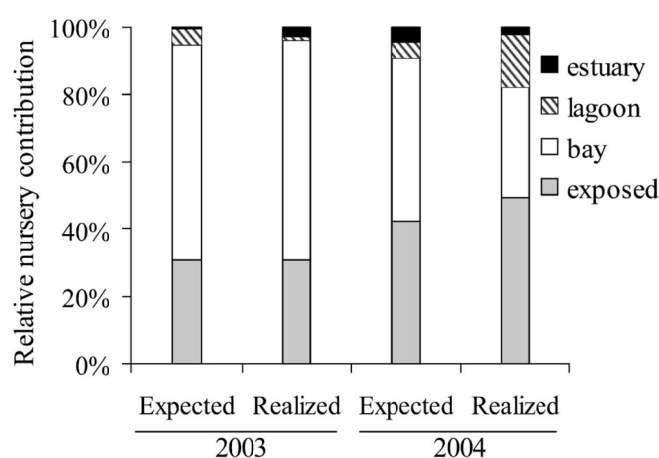
These techniques have probably provided the best evidence so far towards effectively measuring connectivity and identifying nursery role of estuaries. Beyond their isolated value and use, the combined use of several of these natural tags is of increased interest as they provide complementary information on connectivity at different scales and resolutions (Miller et al. 2005, Feyrer et al. 2007, Hedgecock et al. 2007, Abaunza et al. 2008).



**Figure 10.** Predicted estuarine nursery origin of *Solea solea*, *Solea senegalensis*, *Platichthys flesus* and *Dicentrarchus labrax* sampled in each coastal area. Fish were collected in four coastal areas off the Portuguese coast and classified to a nursery estuary based on previously determined multi-element linear discriminant functions generated by juveniles collected in the estuarine nursery areas of: Douro ●, Ria de Aveiro ●, Mondego ○, Tejo ○, Sado ○ and Mira ○. From Vasconcelos et al. (2008).

Some innovative studies have combined assessments of the potential nursery value of an area or habitat and its effective nursery value, as measured for example by the total number of juveniles in the study area and its contribution measured in the habitats used by adults, respectively. Both Fodrie & Levin (2008), studying the nursery value of different types of coastal habitats (estuary, lagoon, bay, exposed) for a marine flatfish in the Pacific coast of the U.S.A., and Vasconcelos (2009), addressing the nursery value of individual estuaries along the Portuguese coast for multiple species, reported a strong match between the results of potential

(i.e. via density, distribution area, growth and survival) and effective metrics (i.e. via direct measures of movement and connectivity with adult stocks) (Figure 11). Both studies found that the importance of a habitat/estuary differed depending on whether the nursery or EJH criterion is considered. Thus both criteria may have distinct significance in providing responses to specific scientific and management objectives. Moreover, Vasconcelos (2009) reported that identifying an area as a nursery or EJH for a single species did not imply that it was of equivalent importance for any of the other remaining present species, highlighting species specific patterns in habitat use and nursery function outcome. Overall, results supported the need for multi-species assessments towards the comprehensive understanding of the relative importance of estuarine habitats for coastal populations and the need for their integrated management.



**Figure 11.** Expected and realized contributions (expressed as a percentage of total) of nursery habitats along San Diego County from 2003 and 2004 in producing the juvenile halibut that successfully advanced to the subadult population sampled in 2005. Expected contributions are based on 2003 (~788,500 juveniles) and 2004 (~826,500 juveniles) field surveys of juvenile halibut distributions and nursery habitat availability. Realized contributions are based on elemental fingerprinting results for 75 individuals that utilized nurseries in 2003 and 129 individuals that used nurseries in 2004. Nurseries are classified as: Exposed; bay; lagoon; and estuary. From Fodrie & Levin (2008b).

Ecosystem based approaches are the ultimate goal in the study of nursery function. However, their application seems unattainable in the short term. The value of such approaches is reinforced by the few available results on nursery function of estuaries simultaneously for several species. Even though not outright ecosystem based approaches, multi-species assessments provide evidence of the outcome of species cumulative and synergistic effects. Potential and magnitude of exchange of individuals between populations may be influenced both by species ecological features and environmental properties. Therefore, connectivity is most surely temporally variable: one measure or one species-specific conceptual frame may not readily apply to all species or times.

Despite the innovative studies addressing the relative contribution of nurseries to coastal populations and the recent reciprocal validation of classic potential juvenile contribution estimates versus effective contribution estimates in the evaluation of the nursery role of different

habitat types (Fodrie & Levin 2008), the direct application of connectivity estimates to ascertain nursery or EJH function can still be considered at an early stage. Moreover, these data do not answer the question of why higher connectivity is observed in some sites or habitats. In most cases, there are no data collected that directly link connectivity measures and the underlying regulating processes; this is quite an overwhelming task. At this moment, it is thus not possible to define the main cause for generating differential contributions: was it due to density, growth, survival or other factors? That which is a simple concept is a challenging one to address.

Nagelkerken (2009) stated that evaluating nursery role per unit area, i.e. as postulated by Beck et al. (2001), is a specific complicating factor. Several confounding issues include the minimum surface area requirements to consider a habitat as a nursery, habitat patchiness or use, and inter-linkages between habitats. In several situations, it may therefore be easier and more valuable for management purposes to calculate total contribution per estuary.

In spite of the widely recognized value of the definitions by Beck et al. (2001) and Dahlgren et al. (2006) concerning nursery function, their relevance and limitations have been argued (Sheaves et al. 2006). The latter state, in particular, that EJH (i.e. Dahlgren 2006) fails to recognize (1) the effects of scale, (2) the importance of complexity and connectivity, (3) the importance of ecosystems, resources and processes in supporting juveniles, and (4) that the value of a nursery ground is a function of the reproductive output of individuals from the nursery and not just the numbers of individuals it provides. Nevertheless, the EJH concept attempts, according to the authors, to offer a framework which simplifies complexity into a quantifiable heuristic towards marine resource management and further research (Layman et al. 2006). Fodrie et al. (2009) elaborated on this issue of how nursery value can be measured, namely from a population perspective. They considered there is a practical limitation in these definitions of nursery as they are solely based on the fate of successful recruits and do not integrate how juvenile growth and mortality affect the population demography and growth rate (i.e. fitness). In their work they consider population growth as a measure of fitness, using cohort analyses and demographic models. A positive overall population growth ( $\lambda > 1$ ) depended critically on the subpopulations of juveniles that utilized coastal embayments (bays, lagoons, and estuaries), as result of juvenile growth and survivorship, even though alternative habitats (exposed coast and coastal embayments) could contribute an approximately equal number of recruits to the adult stock.

In the end, Adams et al. (2006), referring to back reef habitats, identifies the crucial steps that need to be undertaken to develop a comprehensive research strategy to assess nurseries. This crucial four level strategy can easily be adapted to estuaries and estuarine nurseries: (1) building conceptual models to guide research and management; (2) identifying habitat use patterns by juveniles; (3) quantifying connectivity of juvenile and adult populations; and (4) examining the underlying ecological processes that influence levels 2 and 3. In addition, the use of some of the presently discussed natural tags as tools to address connectivity also requires, or will benefit, from targeted research on the premises and specific assumptions that grant their validity (Campana 1999, Elsdon et al. 2008). The conceptual frame and modeling exercises

proposed by Adams et al. (2006) will provide information enabling scientifically-based management decisions, including habitat conservation and restoration towards the safeguard of sustainable fisheries. This framework is vital to prioritize management needs and guide efforts to ecologically important habitats.

## **6. Importance of estuaries for coastal fisheries sustainability: managing and preserving estuarine function**

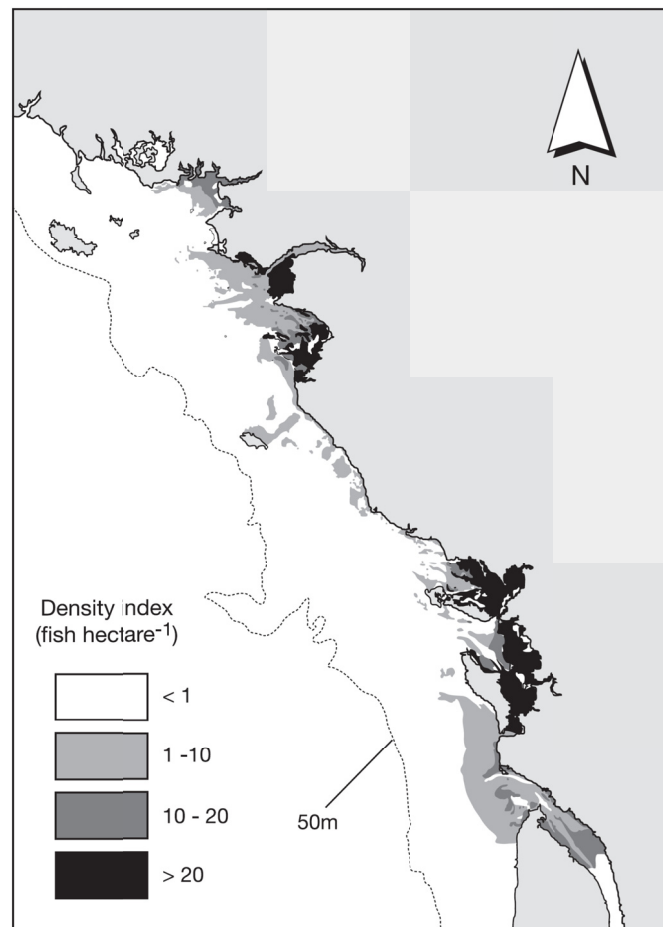
Estuaries are amongst the most productive and valuable ecosystems on earth (Costanza et al. 1997) and this wealth in resources and services is one of the main contributing factors for their widespread human use and consequent degradation. Many studies have highlighted the anthropogenic pressures that affect these systems (Kennish 2002), as well as their end-points in terms of impacts on nursery role for fish species (Vasconcelos et al. 2007). Nevertheless, the economic value of estuaries, namely regarding their acknowledged nursery role and importance towards coastal fisheries, can also be one of the main thrusts towards increasing the interest in the conservation and management of estuarine systems. The key issues explored in this section are related to the connectivity and importance of estuaries to coastal/offshore fisheries. Particular attention will be given to the use of this information in defining management strategies, since it is of major importance to understand how fisheries of multiple commercially important species are affected by changes in estuaries, both of natural or anthropogenic origin. The use of protective habitat management and restoration, as long term management tools, is also addressed. Understanding the impacts and repercussions of human actions is vital to establish guidelines ensuring the sustainable functioning of estuaries.

Coastal habitat degradation or over-fishing seem conspicuous nowadays but one of the main issues raised is how they affect coastal fish populations. The idea that most coastal marine fisheries are estuarine dependent and that effects on the estuaries will impair coastal population is customary; albeit in most cases these direct or indirect links are established with no scientific background or quantitative data. In many cases the influence of estuaries is extrapolated due to broad definitions of estuarine use and dependence. In this context, Ray (1997) argued that the mere occurrence or abundance of species in estuaries could be misleading and that dependence should infer an obligate relationship so that if an estuary or estuaries were, hypothetically, removed these species would be at risk of significant depletion or extirpation.

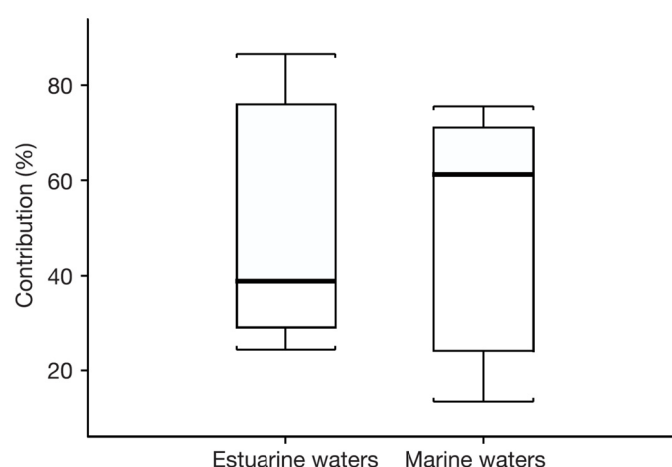
Directly linking coastal fisheries production to estuaries is hard to quantify and most studies focus on correlations and time series analysis, rather than effectively verifying cause-effect relationship and connectivity between estuaries and coastal populations. Studies that have focused on population connectivity have mostly elucidated these aspects indirectly through simulation modeling (e.g. Cowen et al. 2000) or with temporal changes in distributions that are related to abundance and size (Gillanders et al. 2003). Long-term fisheries monitoring data can be a valuable tool for conducting exploratory analyses of connectivity among populations and subpopulations (Kraus & Secor 2005). Nevertheless, the relationship between commercial

catches and mangrove habitats has been found to be highly complex by Blaber (2007), and one of the factors restricting the analysis was the array of highly variable, and often correlated, data such as temperature, rainfall or fishing effort. Moreover, these approaches may be largely influenced by the simultaneous increase throughout the past century in: human induced changes in estuarine environments; fishing pressure; and the efficiency of fishing gears and technology (Blaber 2000).

It has been recognized in many systems that estuarine fish assemblages are dominated by marine migrant species (see section 2) and many of these species represent important coastal fisheries. For instance, along the Portuguese coast five marine fish species show a consistent and abundant use of the estuaries during the juvenile period; and even if numerous other species also occur these five species alone represented in 2008 c. 8% of the national marine fisheries landings in value albeit 1% weight (Vasconcelos et al. 2008). The relative contribution of both coastal and estuarine areas as nursery grounds for a commercially important marine flatfish was analysed via a generalized linear model of habitat suitability, namely to identify the habitats in which juvenile age-0 sole concentrate (Le Pape et al. 2003b) (Figure 12 and 13).



**Figure 12.** Fitted 0-group *Solea solea* density (number of fish per hectare) for mean hydrologic conditions in the Bay of Biscay. From Le Pape et al. (2003b).



**Figure 13.** Contribution (% of total 0-group *Solea solea* in the Bay of Biscay) of estuarine and non-estuarine waters (bar: upper and lower quartiles; whiskers: extreme values; bold line: median value). From Le Pape et al. (2003b).

This quantitative approach emphasized the highly important role of restricted shallow, muddy estuarine areas as nursery grounds of sole in the Bay of Biscay. Moreover, it demonstrated the relation between inter-annual variations in nursery habitat capacity (with respect to estuarine habitat extent) and sole recruitment to adult populations. To address this issue and link juvenile habitat use and availability to sub-adult population size, Fodrie & Levin (2008) took on a different approach. They assigned nursery origins of exposed coasts, bays, lagoons and estuaries for individual sub-adult California halibut via otolith elemental fingerprints and detected a higher relative contribution of coasts and bays.

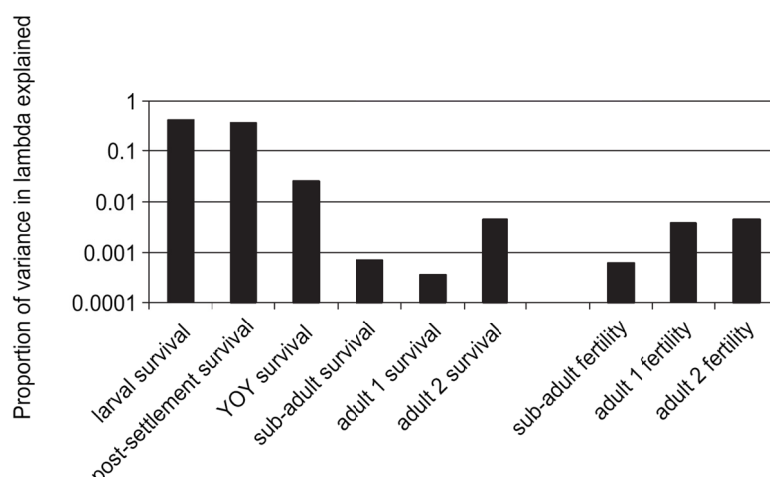
Modeling patterns of connectivity amongst subpopulations, species life-stages and areas/habitats inhabited by them along a geographical region should be a valuable means to assess, and ultimately predict, the role of estuarine nurseries on coastal fisheries and should provide sound basis to optimize fisheries and conservation management strategies. In some cases, model results have provided relevant information for decision-makers to establish marine protected areas and other management priorities (Fogarty & Botsford 2007). For instance, Mumby (2006) generated a connectivity matrix amongst mangrove and coral reefs that facilitated the identification of connectivity between habitats within a dynamic planning environment (e.g. reserve selection algorithms). The challenge regarding modeling is to obtain realistic depictions of connectivity, namely of source and dispersal, linked to the oceanographic processes and life history characteristics of the target species (Werner et al. 2007) in order to test scenarios regarding distinct empirical hypotheses and assumptions. These include responses to fishing effort, habitat protection or conservation efforts (e.g. nursery and spawning areas), or environmental and anthropogenic changes (Guenette et al. 1998, Fogarty & Botsford 2007). Management for fisheries sustainability must necessarily comply with biological,



economic and social objectives as well as with nature's unpredictability which demands for flexible and ecologically adapted strategies. In the end, results should highlight where management and conservation efforts and resources can be invested more wisely towards greater returns.

The traditional fisheries management approach is based on the premise that the productive potential of a fishery is a function, among other factors, of stock size. Stocks or populations are intrinsically linked to the extent to which populations are source, self-replenishing or subsidy-dependant (i.e. sink). Fisheries wise, management has been historically established via spatially and temporally explicit harvesting strategies, including closures of fishing grounds. These practices are still commonly used (see DiBacco et al. 2006). However, throughout the last decades marine protected areas - areas of the ocean protected from one or more forms of human disturbance - have been established both as fisheries management tools and for biodiversity conservation. Expected results of their implementation may include: increases in the abundance and size of target species by lack of fishing pressure; replenishment of adjacent areas and enhancement of local fisheries by spillover effects of adults or larvae dispersal; and enabling ecosystem restoration (Jones et al. 2007). Understanding the patterns of connectivity at ecologically relevant time scales, and for the different life stages (e.g. adult migration, larval dispersal pathways), is a key issue in defining the size, location and number of protected areas (Palumbi 2001). In the context of marine migrant species this may include the establishment of marine and estuarine protected areas.

Inter-annual variations in recruitment produce large variations in fish and invertebrate stocks, creating difficulties for fisheries management. It is widely accepted that variability in year-class strength of many marine migrant flatfish species is generated during the pelagic egg and larval stages, probably by variations in the hydrodynamic circulation and in the mortality rates of eggs and larvae. Density-dependent mortality during the phase shortly after settlement dampens the inter-annual recruitment variability whilst density-dependent growth during juvenile stages has no impact on recruitment variability and there is no evidence of density-dependent effects in the adult phase at present (see review by van der Veer et al. 2000). This has been demonstrated for other biological models of marine migrant species, for instance, by simulation-based sensitivity analysis of a stage-structured matrix model showing that most of the variability in population growth rate ( $\lambda$ ) is explained by larval and juvenile survival rates (Levin & Stunz 2005) (Figure 14). Nevertheless, studies examining the link between recruitment strength and connectivity are scarce mainly because the origins of recruiting larvae have not been evidenced.



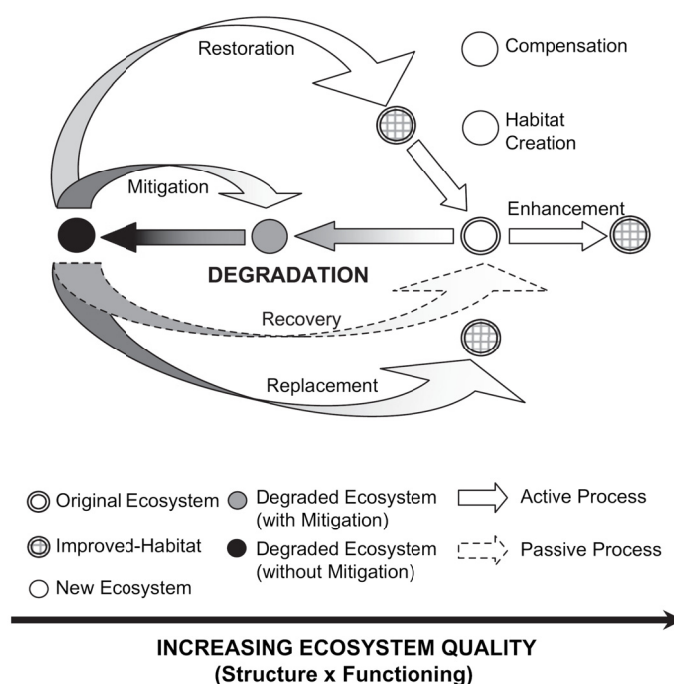
**Figure 14.** Results of a life-stage simulation analysis showing the proportion of variation in  $\lambda$  explained by stage-specific survival rates. From Levin & Stunz (2005).

Coastal fisheries of marine migrant species can only be resilient if they remain connected to sources of replenishment. Conversely, resilience may be diminished by population declines in source areas and loss, degradation or fragmentation of habitats required by the different ontogenetic stages (Mumby et al. 2004). This is likely aggravated by the diversity, intensity and scale of human impacts on marine systems. Whilst measuring connectivity between estuaries and marine areas we should be prepared to find out that connectivity is not identical at all stages (DiBacco et al. 2006). As a means to illustrate what we still need to understand in terms of connectivity in order to ascertain the link between coastal larvae, estuarine juveniles and again coastal adults we can consider for example sea bass *Dicentrarchus labrax* as a model (see Vasconcelos et al. 2008). It is crucial to identify which life stages constitute the critical link where connectivity is maximized. Information on natal origin allied to that of nursery use and gene flow will be fundamental to reveal stock structure and determine if this species presents several subpopulations along the North Eastern Atlantic and the Mediterranean Sea. Young adults along the Portuguese coast showed some level of dispersal once they left the estuary (Vasconcelos et al. 2008), and it would be of interest to assess if this is a generalized trend throughout their distribution range. It has also been shown they perform wide movements in the Bay of Biscay, English Channel and in the coasts of England and Wales (Pickett et al. 2004, Fritsch et al. 2007) in relatively short periods. Thus, until they reach sexual maturity (at age-3 or -4) there is a potential for adults to find spawning grounds distant from their nursery origin. As for larvae, there is hardly any information regarding the origin and dispersal of larvae recruiting to the estuaries. How do these aspects contribute to population mixture or isolation?

Managing a fishery resource within this context may prove to be a conundrum. Stakeholders who invest in nursery habitat conservation and management (i.e. target one life stage) may not get back their investments in terms of fishery resources, if adult organisms naturally disperse away, in particular, to areas under other stakeholders' management or

foreign jurisdiction. The contribution of individual estuaries to fishing stocks and management of such resources and habitats may advocate the creation of internationally discussed and supported management plans, as the one currently under development for the European eel, *Anguilla anguilla* (Council Regulation (EC) 1100/2007). Ultimately, if stocks are found to be composed and replenished by organisms from specific nurseries that contribute to a wide coastal area and are exploited by multi-national fleets, a common protection by those which economically explore them is justified.

The value of protective habitat management and habitat restoration as long-term management tools, towards the mitigation of habitat loss and degradation along with the maintenance of healthy and sustainable fisheries, has been increasingly recognized by fisheries scientists and managers (Turner et al. 1999). These should be viewed as important components of integrated coastal zone and fisheries management programmes. A conceptual framework for restoration and recovery of marine marginal and semi-enclosed areas is presented by Elliott et al. (2007b) along with a review of recent concepts, understanding and experience of the restoration, recovery and human-mediated modification of estuarine, coastal and marine ecosystems (Figure 15).



**Figure 15.** A conceptual model illustrating the nature of natural recovery of a degraded ecosystem and the terms used in human-mediated (active) restoration. The model indicates that habitats can be produced (⊙) which are an improvement on the degraded state but not necessarily to the original state (⊙), whereas other ecosystems (○) are newly created systems. The recovery (light grey dashed arrow) can be to the original state or some distance along that pathway of regaining ecosystem quality. The model emphasises the movement of ecosystems along a continuum (horizontal axis) of ecosystem quality, which combines both structure and functioning, whereas the position of ecosystems in the vertical axis in the model has no meaning. From Elliott et al. (2007b).

Habitat restoration is usually applied when prevention or mitigation of adverse impacts fails (Turner et al. 1999). Often ecosystems do not recover from anthropogenic disturbance without additional management or manipulation (Pratt 1994) and the original environmental conditions will need to be re-established before habitat restoration can be successfully achieved (Turner et al. 1999). Overall, habitat restoration seeks to reinstate important habitat structures and their ecological function to a level, based on feasibility and historical information, which ensures the system's long-term productivity (Turner et al. 1999). Restoration or creation usually consists of (1) managed realignment – involving, for instance, the retreat of sea defenses by breaching existing dykes and allowing saltmarsh to develop; (2) managed advance - gaining new area by stimulating sedimentation, for example, by creating structures to simulate low energy accreting regimes; (3) managed stability - to compensate erosion locally through soft engineering practices (Cattrijsse et al. 2002).

Even though habitat restoration or creation has been recently addressed and numerous projects have been proposed to restore large scale habitat losses (see Cattrijsse et al. 2002), it still has had only a limited application. Some examples are: the creation of artificial creeks; the redesign of sections of shoreline to create intertidal zones; the managed inundation of reclaimed agricultural land and reversion to intertidal mudflats and saltmarshes; and the production of reed beds to compensate loss of mudflats.

Restoring particular estuarine habitats (e.g. seagrass or salt marsh) is expected to benefit those species that are dependent on their resources (McCay & Rowe 2003). Habitat-directed management and conservation is particularly interesting concerning the nursery role of estuaries, since habitats with an acknowledged higher nursery role can be identified and efforts can be directed at them (Beck et al. 2001). Via habitat restoration models based on food chain transfers, McCay & Rowe (2003) determined that the restoration of seagrass beds would be more productive than salt marsh restoration in southern New England and, likely so, in other temperate areas. This novel approach provides a methodology to estimate the scale of a restoration project that will provide production (ecological services) equivalent to the losses of organisms at multiple trophic levels and is applicable to natural resource damage assessments as well as other environmental assessments. Nevertheless, the extreme importance of habitat diversity, patchiness and connectivity within estuaries, in addition to their availability, should not be overlooked (Sheaves et al. 2006).

Despite the potential interest of restoration approaches in estuarine environments, their true value is controversial (Wolanski et al. 2004). Few studies have directly evaluated the effectiveness of these measures or the habitats' true value, for instance in terms of function or services, or their use by resources such as benthos and fish (e.g. Hampel et al. 2003). Reviewing the restoration of former salt hay farms to functioning salt marshes, Able et al. (2008) documented their functional success for fauna, especially fishes, both resident and transient, particularly creating high-quality juvenile fish habitat with enhanced feeding and growth conditions (Miller & Able 2002, Nemerson & Able 2005). Nevertheless, Cattrijsse et al. (2002) question if the authentic communities could ever be restored and argue that mitigation or

compensation projects can be inadequate in replacing the original habitat and that habitat creation or restoration seldom mitigates habitat loss. They advocate that species-rich, mature and complex habitats that have existed for a long time are not readily replaceable by simple re-vegetation or re-creation; and that a newly created habitat is of low value and it will take decades or centuries to reach the state of the genuine habitat and its community. Even so, the variable and often ephemeral nature of estuarine habitats and communities may denote that this is less of a problem than in more stable environments. This can be particularly true for mudflat habitats. These habitats are characterized by low biodiversity assemblages able to withstand changes in geomorphology and may potentially evolve to a mature system in a relatively shorter period, whereas re-vegetated seagrass or saltmarsh habitats will take more time to evolve as they are of a more static nature. In the end, habitat preservation and conservation should always be the preferred option (Elliot 1995, Fonseca et al. 1998).

Ecohydrology, as a scientific concept, recognises that the present practice of relying nearly exclusively on engineering fixes to solve environmental problems is failing to restore the aquatic environment to a level that can sustain presently demanded life quality standards (Chícharo & Chícharo 2006). However, its development is still incipient. An ecohydrology model integrating physical, chemical and biological processes has demonstrated that the sustainability of estuarine ecosystems, in terms of sediment distribution, erosion control, nutrients and different trophic web components in the estuary, as well as in the coastal zone, can be achieved by controlling the volume and timing of freshwater discharge (Chicharo & Chicharo 2006, Wolanski et al. 2006). Moreover, it allowed the prediction of the ecosystem's health and the consequences of human activities throughout the river catchment (e.g. downstream irrigation farming, downstream saltmarsh reclamation by urban developments and flow regulation).

As pointed out by Elliott et al. (2007b), whereas some ecological concepts related to restoration are well understood, for example the nature of ecosystem structure and functioning, others, such as carrying capacity, resilience and ecosystem goods and services, are still poorly quantified for the marine and estuarine environments. Linking these ecological concepts and the management framework is also relatively recent but it is of paramount importance towards a holistic approach to understand, manage and manipulate these environments.

Knowledge of the impacts and interactions of human activities and their direct and indirect influence in estuaries, namely in terms of their importance as nursery areas and on other ecological functions and services, is therefore vital towards establishing effective management efforts, including where necessary, habitat restoration programmes. Management and conservation efforts must target the sustainable functioning of estuaries and it is fundamental to address this issue in a comprehensive manner (Vasconcelos et al. 2007). Successful application involves evaluating uncertainty, incorporating place-based information, and engaging diverse constituencies in the planning process. This implies combining knowledge on the intensity of human activity and impacts in addition to the cultural and socio-economic factors relevant to the restoration goals (Baird 2005). Worldwide there is a lack of long term data series and the window of opportunity to gather baseline data is narrowing whilst estuarine habitats are

being altered at a rapid rate (Peterson 2003). Studies covering and integrating a wide field of subjects, such as the ecohydrology approach, are a valid tool towards a comprehensive view of estuaries globally. Ultimately, this should provide the means to assess changes, predict trends and prevent future degradation whilst establishing viable management plans.

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## CHAPTER 3

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### **Effects of temperature, salinity and water composition on otolith elemental incorporation of *Dicentrarchus labrax***

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## Effects of temperature, salinity and water composition on otolith elemental incorporation of *Dicentrarchus labrax*

**Abstract:** Elemental composition of fish otoliths provides a valuable means to retrospectively determine a fish's environmental history over time. However, to reconstruct patterns of fish movement and migration using otolith chemistry it is essential to establish the link between changing environmental variables, in particular, temperature, salinity and elemental concentration, and the concentration of elements within otoliths. Using a controlled laboratory experiment we investigated how otolith chemical composition of Sr:Ca and Ba:Ca was affected by temperature, salinity and ambient elemental concentration in juvenile sea bass *Dicentrarchus labrax*. A total of 18 treatments representing all combinations of temperature (21, 25 °C), salinity (10, 20, 30) and ambient water concentrations (Low, Medium, High) were assessed. Otolith elemental composition of Sr:Ca and Ba:Ca were positively related to ambient water concentration. Consistent interactions between salinity and concentration were found for otolith composition, as well as elemental incorporation, for both elements. Positive effects of temperature on elemental composition and incorporation were also detected. The partition coefficient of Ba ( $D_{Ba}$ ) initially decreased with increased water concentration but increased at the highest level of added elemental concentration. Across salinity gradients,  $D_{Ba}$  increased whilst distinct patterns in variation of  $D_{Sr}$  occurred within water concentration treatments. Our data highlight the importance of validation experiments to decipher species-specific patterns in otolith chemistry, particularly if the goal is to reconstruct fish life histories and determine fish movement patterns and habitat use.

**Keywords:** Otolith chemistry; partition coefficient; strontium; barium; salinity; temperature

### 1. Introduction

Otoliths are valuable tools to assess movement and life history characteristics of fish. Specifically, elemental composition of otoliths has provided a wealth of information in a wide range of topics and has been used to assess migration patterns (e.g. Tsukamoto & Arai 2001, Crook et al. 2006, Walther et al. 2011), fish stock structure (e.g. Campana et al. 2000, Rooker et al. 2003, Ferguson et al. 2011), natal origin or nursery habitats (e.g. Brown 2006, Vasconcelos et al. 2007, Tanner et al. 2012) and determine connectivity, by retrospectively assigning adults to their areas of origin (e.g. Thorrold et al. 2001, Gillanders 2005, Vasconcelos et al. 2008). These applications are possible because otoliths are metabolically inert and not resorbed through time, they grow continuously throughout the life of the fish and they form daily and annual growth patterns that allow information from the otolith to be linked to a time component, resulting in a time resolved elemental tag.

Although it is not necessary to fully understand the mechanisms regulating elemental incorporation to use otoliths as group-specific natural tags for stock discrimination or connectivity studies (Thorrold et al. 1998, Campana 1999, Elsdon et al. 2008), the reconstruction of environmental histories of fish and description of fish movements and differential habitat use depends on effectively coupling environmental changes with otolith chemistry (Elsdon et al. 2008). Ultimately, such applications rely on establishing predictable responses of otolith chemistry to changes in environmental settings, such as ambient elemental

composition (water chemistry), salinity and temperature. Moreover, given the presence of species specific effects on otolith chemistry (e.g. Gillanders & Kingsford 2003, Reis-Santos et al. 2008, Melancon et al. 2009) validation studies should be carried out on the species and age groups of interest to provide an appropriate framework for future field applications and movement reconstructions.

Differentiation between freshwater and marine residency has been successfully accomplished based on otolith chemistry. As strontium (Sr) and barium (Ba) water concentrations tend to be inversely related in coastal systems, with lower Sr and higher Ba in freshwater end-members [though see Kraus & Secor (2004) as exceptions occur]; these elements have been used in combination to unravel environmental and migratory histories (Macdonald & Crook 2010, Walther & Limburg 2012). However, discriminating habitat use over finer salinity scales and reconstructing movements within estuaries poses a wider challenge. Estuaries are complex and dynamic environments, with movements of fish occurring within and across gradients of salinity, temperature and also water chemistry, as a result of varying physical and chemical properties due to the mixing of marine and freshwater inputs. Before linking otolith chemistry to a fish's life history or differential habitat use patterns, we need to evaluate the degree to which they can be accurately determined (Martin & Wuenschel 2006, Hicks et al. 2010, Macdonald & Crook 2010, Miller 2011).

Laboratory validations are an important step towards understanding abiotic (temperature, salinity, water concentration) as well as biotic effects (physiology) on the incorporation rate of elements into otoliths. Several studies have used controlled laboratory experiments to assess the relationship between environmental variables and otolith elemental composition, including for elements such as Sr and Ba (e.g. Bath et al. 2000, Elsdon & Gillanders 2002, 2004, Miller 2011). Effects of temperature, ambient water concentrations and salinity on otolith chemistry have been examined; however, most studies have focused on single factor assessments and do not encompass the natural conditions or the scale of physical and chemical variation that fish encounter in the wild.

Given that estuaries often vary in terms of environmental features (e.g. changes in salinity due to increased seawater may coincide with changes in temperature and water chemistry), single factor reconstructions may be insufficient and provide erroneous interpretations of fish movement patterns (Elsdon & Gillanders 2004). Moreover, variation in temperature, salinity and ambient Sr and Ba concentration may vary independently, but they are also known to co-vary, which could also have a major role in confounding otolith data interpretations if multiple environmental factors are not taken into consideration (Elsdon & Gillanders 2002).

A better understanding of the mechanisms affecting otolith elemental incorporation and composition is essential prior to field application, and the lack of information on the interactive effects of temperature, salinity and ambient elemental concentrations on otolith chemistry limits our interpretations of fishes' environmental histories and differential habitat use patterns or migrations (Elsdon et al. 2008, Miller 2011). Therefore, we designed a controlled laboratory experiment that simultaneously manipulated multiple environmental variables to determine their

influence on otolith chemistry. Specifically, we evaluated the effects of water chemistry (Sr and Ba concentrations) and temperature across an estuarine salinity gradient on otolith composition of juvenile *Dicentrarchus labrax*.

## 2. Material and methods

### 2.1. Study species

The sea bass *Dicentrarchus labrax* (Linnaeus 1758) is a commercially important marine migrant species that uses estuaries throughout its juvenile life history. Adults inhabit the marine environment, where they spawn, and post-larvae / early juveniles enter estuaries in spring. Within estuaries, juvenile *D. labrax* can tolerate a wide range of temperatures and salinities occupying a variety of habitats (e.g. Vasconcelos et al. 2010). Thus, this species is a good model for examining issues concerning the influence of multiple environmental factors on otolith element incorporation.

### 2.2. Experimental design

Juvenile *D. labrax* of approximately 25 mm in total length were obtained from a hatchery and held in a controlled temperature room in 100 L tanks, at 21 °C and 20 salinity equipped with aeration and filtration. The temperature and salinity of the water in each of these holding tanks was gradually adjusted over a five day period to acclimatize fish to one of the water temperature (21, 25 °C) and salinity (10, 20, 30) experimental treatment combinations. These temperature and salinity treatments, as well as three water concentration treatments (ambient, also referred to as Low; Medium and High, which consisted of 4x and 10x enhanced levels of both Sr and Ba, respectively) were used as categorical variables in an orthogonal design to test the relative and interactive effects of temperature, salinity and water elemental composition on otolith chemistry. Three replicate tanks for each of the 18 different treatment combinations were used (54 tanks in total). Levels for these different treatments were chosen as they represent the range of environmental conditions that wild age-0 *D. labrax* may experience during their juvenile stage in estuaries.

After the initial acclimatization to the experimental conditions in one of the holding tanks, fish were randomly allocated to 20 L experimental tanks at densities of 7 to 8 fish per tank. Each tank was fitted with adequate aeration and a clear lid to minimize evaporation. Electric aquarium heaters were used to control temperatures within individual tanks. Salinity was regulated by mixing Instant Ocean synthetic sea salt with deionised water, and elevated concentrations of Sr and Ba were manipulated by addition of standard solutions of  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$  and  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ , such that both elements were spiked at the same level in each treatment, regardless of salinity, ensuring a constant spike volume (Elsdon & Gillanders 2003, Miller 2009, Webb et al. 2012). Sr and Ba were spiked independently of salinity as previous studies have shown that salinity and ambient water Sr and Ba concentrations may be decoupled in nature (e.g. Kraus & Secor 2004, Dorval et al. 2007, Tanner et al. 2013). In addition, we chose to use similar enhancements for both Sr and Ba to reduce potential interactions between elements (e.g. facilitation) (de Vries et al. 2005).



Throughout the duration of the experiment, *D. labrax* were fed specialized fish food (same as the one produced and given at the hatchery, Maresa – Mariscos de Esteros) to apparent satiation. Any accumulated detritus on the bottom of the tanks was siphoned away daily. Water temperature and salinity were monitored daily. Approximately 50 % of the water in each treatment tank was replaced regularly to maintain water quality and prevent the build-up of metabolic wastes. Photoperiod was set at a 12 h day / 12 h night cycle. Fish were reared in the treatment tanks for 32 days. After this period of exposure to the experimental conditions fish were removed from their tanks, immersed in ice slurry and stored frozen.

All plastic- and glassware, including holding and experimental tanks, as well as all the material used for water and otolith sample preparation, collection and storage, were washed in 10 % analytical grade nitric acid ( $\text{HNO}_3$ ) (see Vasconcelos et al. 2007 for more details).

### **2.3. Water elemental concentrations**

Water samples were taken from each tank over the course of the experiment at approximately weekly intervals ( $n = 4$ ). Samples were collected in duplicate using polypropylene syringes and filtered ( $0.2 \mu\text{m}$ ) into 8 ml vials, acidified (1:50) with concentrated ultrapure  $\text{HNO}_3$  and stored at  $4^\circ\text{C}$  until analysis. Calcium (Ca) concentration in the water samples was determined using a Varian 730-ES ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer), whilst Sr and Ba concentrations were determined using a Perkin Elmer Elan DRC II ICP-MS (Dynamic Reaction Cell Inductively Coupled Plasma – Mass Spectrometer). Internal standards of yttrium (Y) and indium (In) were used to correct for drift of the ICP-AES and ICP-MS, respectively. Samples were all above detection limits for all elements. The concentration was quantified by using an external calibration curve with a correlation coefficient of at least 0.999. Sample dilution (if any) was based on volume. All water samples were analysed at the National Measurement Institute (NMI), Australia.

Mean water elemental concentration per tank was calculated using the water samples collected throughout the experiment. The concentrations of Sr and Ba in water ( $\mu\text{g} \cdot \text{L}^{-1}$ ) were standardised to Ca, and expressed as an element to Ca ratio ( $\text{Me}:\text{Ca}_{\text{Water}}$ ). All further data analyses were carried out on the  $\text{Me}:\text{Ca}_{\text{Water}}$  data.

### **2.4. Otolith elemental concentrations**

Five fish per tank were randomly selected for analysis (total of 15 fish per treatment). Otolith sample preparation and analysis followed the methods described in Reis-Santos et al. (2012). Sagittal otoliths were extracted, using plastic forceps, washed and cleaned of adhering tissue with ultra-pure water and allowed to air dry in microcentrifuge tubes in a positive pressure laminar flow hood. The right otolith of each fish was embedded in EpoFix resin (Struers), spiked with indium ( $^{115}\text{In}$ ) at 30 ppm to allow discrimination between otolith material and resin during analysis. Otoliths were sectioned transversely through the nucleus using a low-speed saw (Buehler Isomet). Resulting otolith sections were polished to c.  $250 \mu\text{m}$  with lapping film ( $30 \mu\text{m}$ ,  $9 \mu\text{m}$  and  $3 \mu\text{m}$  grit size successively, using ultrapure water to remove surface contamination)

and mounted on glass slides with indium-spiked thermoplastic glue (CrystalBond 509). Slides were then cleaned, sonicated and triple-rinsed with ultrapure water, dried under a laminar flow hood and stored individually in sealed plastic bags.

A New Wave 213 nm UV high performance (Nd:YAG) laser microprobe coupled to an Agilent 7500cs inductively coupled plasma mass spectrometer (ICP-MS) was used to quantify  $^{43}\text{Ca}$ ,  $^{88}\text{Sr}$  and  $^{138}\text{Ba}$  concentrations in otoliths. Ca was used as an internal standard to correct for variation in ablation yield between samples.  $^{115}\text{In}$  was also measured but solely as a marker to discriminate between spiked resin, or CrystalBond, and otolith matrices. Sectioned otoliths were analysed using spot analysis with 25  $\mu\text{m}$  beam diameter on the outer edge of the otoliths to ensure that the material analysed was laid down during exposure to the experimental conditions - applied spot size encompassed c. 5 – 8 days of otolith growth (e.g. Gutiérrez & Morales-Nin 1986). Elemental data were collected in the same region of the dorsal quadrant for each otolith. A reference standard, NIST 612 (National Institute of Standards and Technology), was analysed at the start and end of each session and after every 10 otoliths to correct for mass bias and machine drift. External precision (% relative standard deviation) was determined based on a calcium carbonate certified reference material, MACS-3 (United States Geological Survey), and was 2.4 % and 2.5 % for Sr and Ba, respectively. All acquired data were above detection limits, set as  $3\sigma$  above background counts. Data reduction, including background corrections, mass count data conversion to concentrations (ppm) and limits of detection were all performed for each individual sample using Glitter software (GEMOC, Macquarie University, Sydney, Australia).

Otolith elemental concentration data were converted to molar concentrations and standardised to calcium ( $\text{Me}:\text{Ca}_{\text{Otolith}}$ ), as elements such as Sr and Ba are likely to substitute for Ca in the otoliths of fish (Campana 1999). All further data analyses were carried out on the  $\text{Me}:\text{Ca}_{\text{Otolith}}$  data.

## 2.5. Data analyses

Mean  $\text{Me}_{\text{Water}}$ ,  $\text{Me}:\text{Ca}_{\text{Water}}$ ,  $\text{Me}:\text{Ca}_{\text{Otolith}}$  and partition coefficients ( $D_{\text{Me}}$ ) were calculated per tank. The three tank averages were used as replicates for each experimental treatment and values per treatment plotted. The relationship between  $\text{Me}:\text{Ca}_{\text{Water}}$  and  $\text{Me}:\text{Ca}_{\text{Otolith}}$  was also investigated using the measured values for Me:Ca in both the water and otoliths.

Partition coefficients provide a useful metric to compare elemental incorporation across experimental conditions as they describe otolith chemical concentrations relative to water chemical concentration.  $D_{\text{Me}}$  was calculated for each tank by dividing the element:Ca ratio measured in an otolith by the mean element:Ca ratio measured in each experimental tank (Morse & Bender 1990).

Statistical analyses were conducted using PRIMER 6 / PERMANOVA. Differences among treatments in  $\text{Me}:\text{Ca}_{\text{Water}}$ ,  $\text{Me}:\text{Ca}_{\text{Otolith}}$  and  $D_{\text{Me}}$ , were analysed separately using a three way permutational univariate analysis of variance (PERMANOVA); these data were treated as continuous variables. Permanova has the advantage over traditional ANOVA as the null

distribution of the test statistic does not require formal assumptions of multivariate homoscedasticity and normality (Anderson 2001). Euclidean distance dissimilarity matrices and unrestricted permutations were used. Variables were  $\log(x+1)$  transformed. Salinity, temperature and water elemental concentration were treated as fixed categorical factors and replicate tanks included as a random factor (nested within all three fixed factors). If significant differences were detected, *post-hoc* pairwise tests were used to determine which treatments or tanks differed.

### 3. Results

#### 3.1. Rearing conditions

Temperature and salinity reflected the desired treatment levels with little variation throughout the experimental period (Table 1). Concentrations of Sr, Ba and Ca increased with salinity levels. Manipulations of water chemistry approximated the expected results, with Sr and Ba concentrations increasing with experimental spiking regimes, whilst Ca concentrations remained similar for each salinity level (Table 1). The medium and high spiking treatments were based on values at the low salinity (10) hence values at 20 and 30 salinity did not always equate exactly to 4x and 10x concentrations. Nevertheless, the low, medium and high concentration treatments clearly differed within each salinity.

**Table 1.** Summary of rearing conditions of *Dicentrarchus labrax* in each experimental treatment. Shown are mean (and standard error) of temperature, salinity, and water Sr, Ba, and Ca concentrations. Standard error of 0.0 indicates no variation or variation less than 0.09

Treatment			Salinity	Temperature	Sr	Ba	Ca
Salinity	Temperature (°C)	Concentration		(°C)	(mg L <sup>-1</sup> )	(µg L <sup>-1</sup> )	(mg L <sup>-1</sup> )
10	21	L	10.0 (0.0)	21.04 (0.10)	2.39 (0.14)	8.31 (0.74)	131.00 (9.13)
		M	10.1 (0.0)	20.97 (0.09)	9.76 (0.18)	28.14 (0.59)	110.00 (4.92)
		H	10.0 (0.1)	20.99 (0.04)	22.50 (0.31)	59.86 (0.98)	104.71 (5.39)
	25	L	10.0 (0.0)	24.89 (0.07)	2.07 (0.06)	7.41 (0.55)	115.23 (3.91)
		M	10.1 (0.1)	24.85 (0.07)	9.55 (0.14)	28.40 (0.32)	103.13 (3.57)
		H	10.1 (0.0)	24.87 (0.03)	22.47 (0.49)	57.20 (1.79)	113.40 (5.85)
20	21	L	20.0 (0.1)	21.08 (0.05)	4.04 (0.10)	13.29 (0.42)	227.14 (5.97)
		M	20.0 (0.0)	21.01 (0.09)	11.43 (0.20)	34.36 (0.59)	220.00 (6.38)
		H	20.2 (0.1)	20.87 (0.03)	24.85 (0.22)	65.54 (0.95)	210.77 (8.04)
	25	L	20.1 (0.0)	24.76 (0.13)	4.08 (0.20)	13.50 (0.56)	233.33 (8.82)
		M	20.2 (0.1)	25.16 (0.15)	12.33 (0.42)	37.17 (1.35)	255.00 (9.57)
		H	20.1 (0.0)	24.75 (0.10)	25.88 (0.77)	66.75 (1.46)	245.00 (8.45)
30	21	L	30.1 (0.2)	21.31 (0.05)	5.54 (0.13)	18.00 (0.54)	328.33 (8.15)
		M	30.2 (0.1)	21.03 (0.02)	12.60 (0.37)	37.70 (1.13)	319.00 (12.86)
		H	30.2 (0.0)	20.65 (0.03)	26.45 (0.45)	70.09 (0.98)	325.45 (6.79)
	25	L	30.0 (0.1)	24.88 (0.18)	5.64 (0.17)	18.13 (0.58)	338.75 (10.25)
		M	30.2 (0.1)	24.98 (0.12)	14.17 (0.31)	41.67 (0.99)	368.33 (7.92)
		H	30.1 (0.1)	25.16 (0.14)	28.43 (0.61)	74.57 (1.41)	352.86 (10.63)

L = Low; M = Medium and H = High concentration treatments

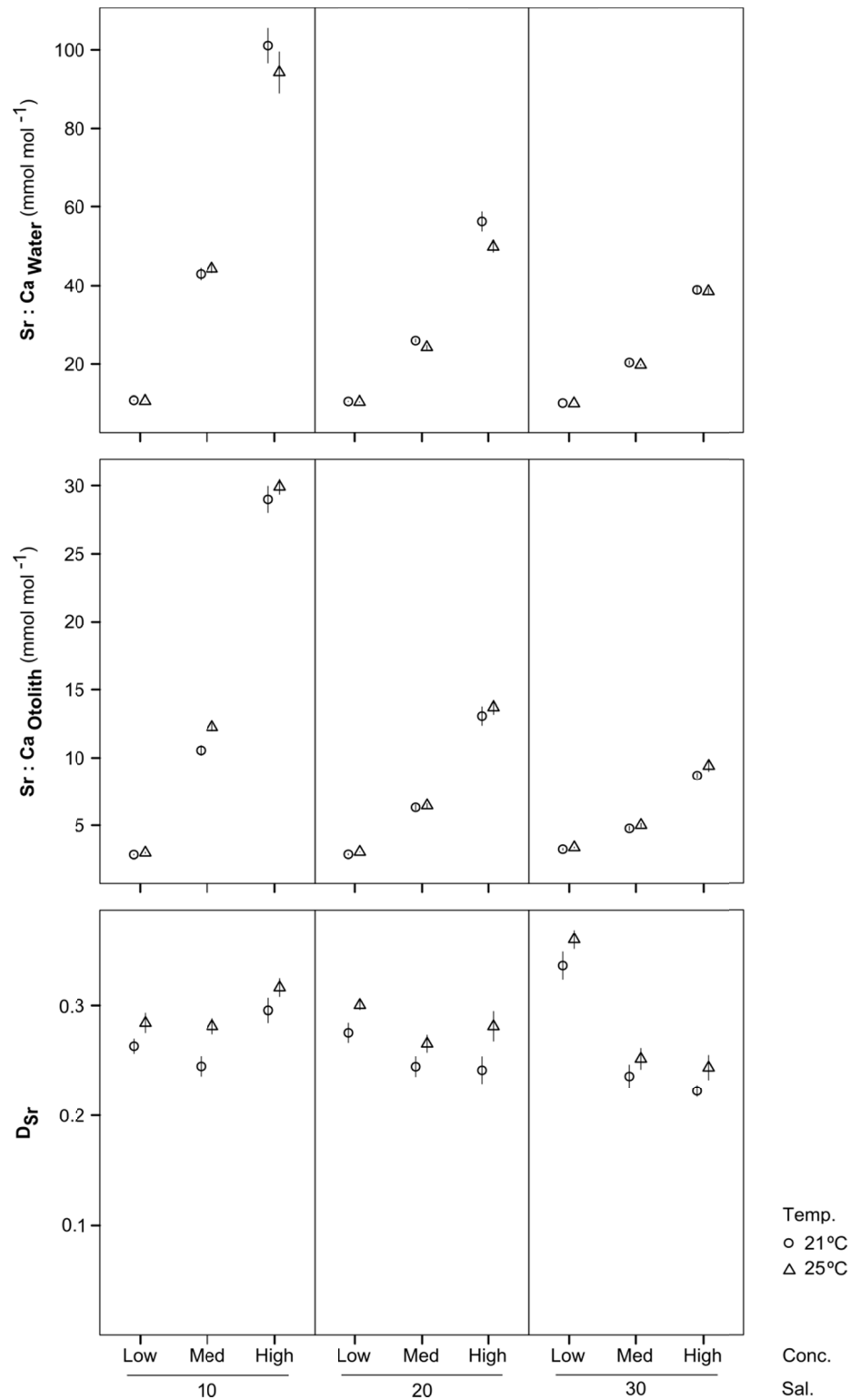
Me:Ca<sub>Water</sub> differed significantly among treatments, but not among tanks. An interaction between salinity and elemental concentration was found for both water Sr:Ca and Ba:Ca (Table 2), whereby lower salinity treatments with spiked elemental concentrations had significantly higher Sr:Ca and Ba:Ca than those at higher salinities (Figures 1, 2). For Ba:Ca, an interaction between salinity, temperature and concentration was also found in the rearing water, with *post hoc* tests indicating that Ba:Ca decreased significantly with temperature in the high concentration treatment.

**Table 2.** Results of permutational analysis of variance (PERMANOVA) examining the effects of salinity (S), temperature (T) and concentration (C) on Sr:Ca and Ba:Ca in the rearing water of *Dicentrarchus labrax*. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001

		Sr:Ca	Ba:Ca
Source of variation	df	MS	MS
<b>Water</b>			
Salinity	2	6.387***	6.656***
Temperature	1	0.066**	0.056**
Concentration	2	51.588***	42.206***
Salinity x Temperature	2	0.015	0.017
Salinity x Concentration	4	1.299***	1.124***
Temperature x Concentration	2	0.017	0.041
Salinity x Temperature x Concentration	4	0.009	0.020*
Tank (S x T x C)	36	0.006	0.006
Residual	146	0.013	0.014

### 3.2. Otoliths

A significant interactive salinity x concentration effect was found for otolith Sr:Ca (Table 3, Figure 1). Even though there was an increase in otolith elemental concentration with increasing water concentration, overall the magnitude of variation differed among salinities, with differences more pronounced at the lower salinities (Figure 1). *Post hoc* pairwise tests found significant differences among all treatments, with the exception of otolith Sr:Ca at salinities 10 and 20, within the Low concentration level. In general, enhancing Sr in water resulted in increased otolith concentrations of Sr:Ca but within each of the enhanced concentration treatments a decrease in otolith Sr:Ca with salinity was observed. At Low concentration a small but significant increase in otolith Sr:Ca was observed for 30 salinity. A significant temperature effect was also found for otolith Sr:Ca, with increased otolith Sr:Ca concentration at 25 °C. A significant tank effect was also detected for otolith Sr:Ca. *Post hoc* tests found variation among tanks within five treatments, mostly at High concentration treatments. These are likely due to small differences between rearing conditions, namely water elemental concentration in replicate tanks. A significant positive linear relationship was found between Sr:Ca in otoliths and Sr:Ca in water ( $R^2 = 0.979$ , Figure 3).



**Figure 1.** Mean and standard error bars of Sr:Ca<sub>Water</sub>, Sr:Ca<sub>Otolith</sub>, and  $D_{Sr}$  across concentration (Low, Medium, High), salinity (10, 20, 30) and temperature (21 °C - open circles, 25 °C - open triangles) experimental treatments of *Dicentrarchus labrax*.

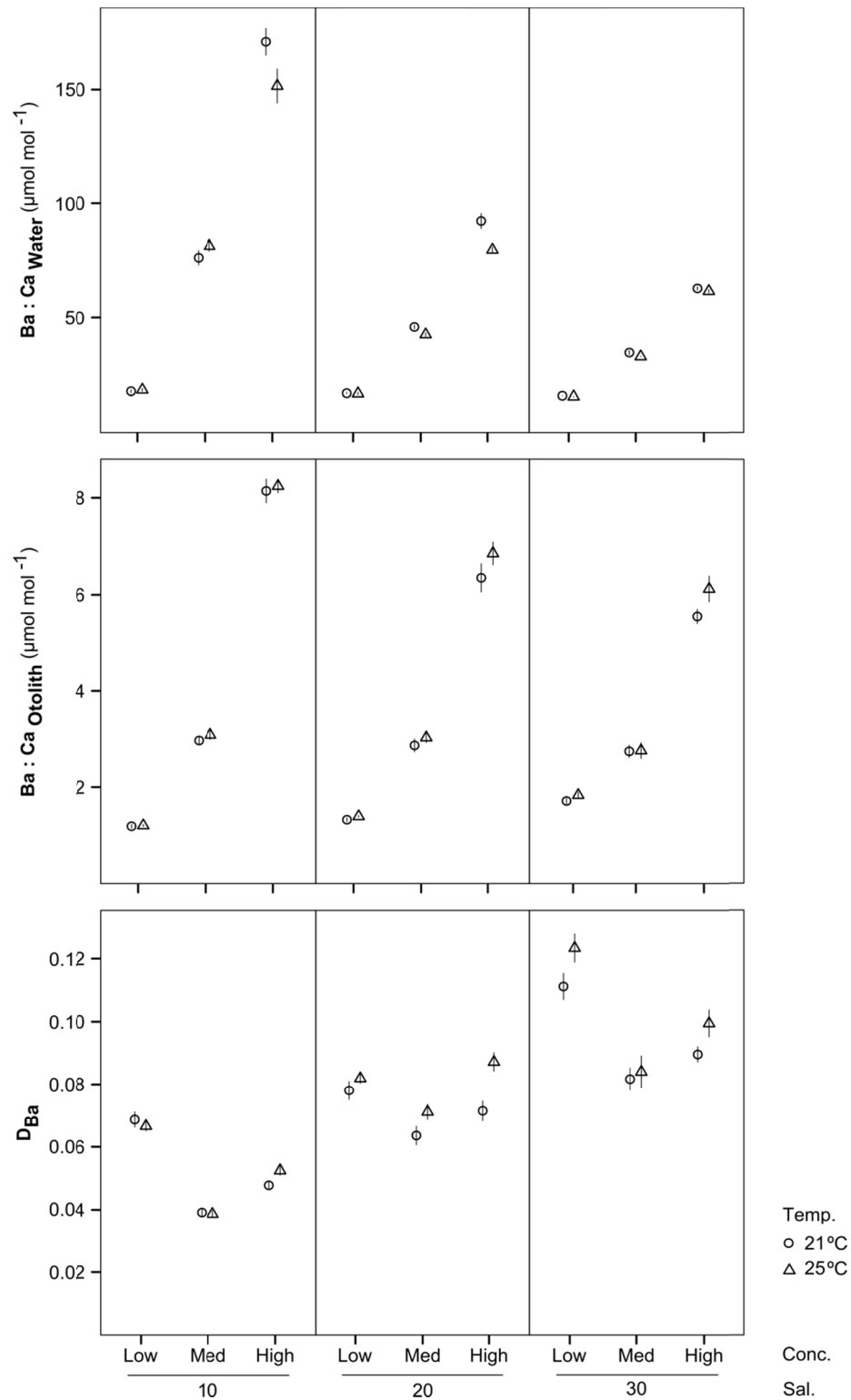
**Table 3.** Results of permutational analysis of variance (PERMANOVA) examining the effects of salinity (S), temperature (T) and concentration (C) on otolith Sr:Ca and Ba:Ca of *Dicentrarchus labrax*.

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

		Sr:Ca	Ba:Ca
Source of variation	df	MS	MS
Otolith			
Salinity	2	9.088***	0.072*
Temperature	1	0.234**	0.095*
Concentration	2	50.237***	28.297***
Salinity x Temperature	2	0.006	0.006
Salinity x Concentration	4	3.257***	0.604***
Temperature x Concentration	2	0.003	0.013
Salinity x Temperature x Concentration	4	0.017	0.006
Tank (S x T x C)	36	0.027***	0.019*
Residual	216	0.012	0.010

A significant salinity x concentration interaction, as well as a significant temperature effect were also found for otolith Ba:Ca (Table 3, Figure 2). Overall, patterns of variation in Ba:Ca<sub>Otolith</sub> along the salinity gradient for each concentration level were more complex. *Post hoc* tests indicated that at Low concentration treatments otolith Ba:Ca<sub>Otolith</sub> differed amongst the three salinity levels, whilst at Medium concentration Ba:Ca<sub>Otolith</sub> was significantly lower at 30 salinity, and at High concentration Ba:Ca<sub>Otolith</sub> decreased significantly with salinity. A significant tank effect in otolith Ba:Ca was also found, with significant variation identified for replicate tanks among six treatments, mostly at High concentration treatments. As with Sr:Ca, there was a positive effect of Ba:Ca water concentrations on otolith Ba:Ca, although the significant interaction between water concentrations and salinity was reflected in a poorer fit ( $R^2 = 0.795$ ) (Figure 2, 3).

$D_{Sr}$  ranged from  $0.22 \pm 0.02$  to  $0.36 \pm 0.03$ , with overall strontium incorporation significantly greater at 25 °C than at 21 °C (Figure 1, Table 4). A salinity x concentration interaction was also detected (Table 4). Within each concentration treatment, trends in  $D_{Sr}$  varied with salinity and increased at Low concentration, but decreased at Medium and High concentrations. For barium,  $D_{Ba}$  ranged from  $0.040 \pm 0.001$  to  $0.123 \pm 0.004$ . A significant overall effect of increasing  $D_{Ba}$  with temperature was also detected together with an interaction between salinity and elemental concentration (Figure 2, Table 4).  $D_{Ba}$  increased with salinity, both overall and within each of the three levels of elemental concentration. Within each salinity level,  $D_{Ba}$  decreased from Low to Medium concentrations but then increased for High concentration treatments (Figure 2). As Ba:Ca<sub>Water</sub> decreased with salinity within each concentration treatment, a pattern of higher elemental incorporation with decreasing water concentration could also be established. Tank effects were also found for  $D_{Sr}$  and  $D_{Ba}$  in five treatments, mostly at High treatments.

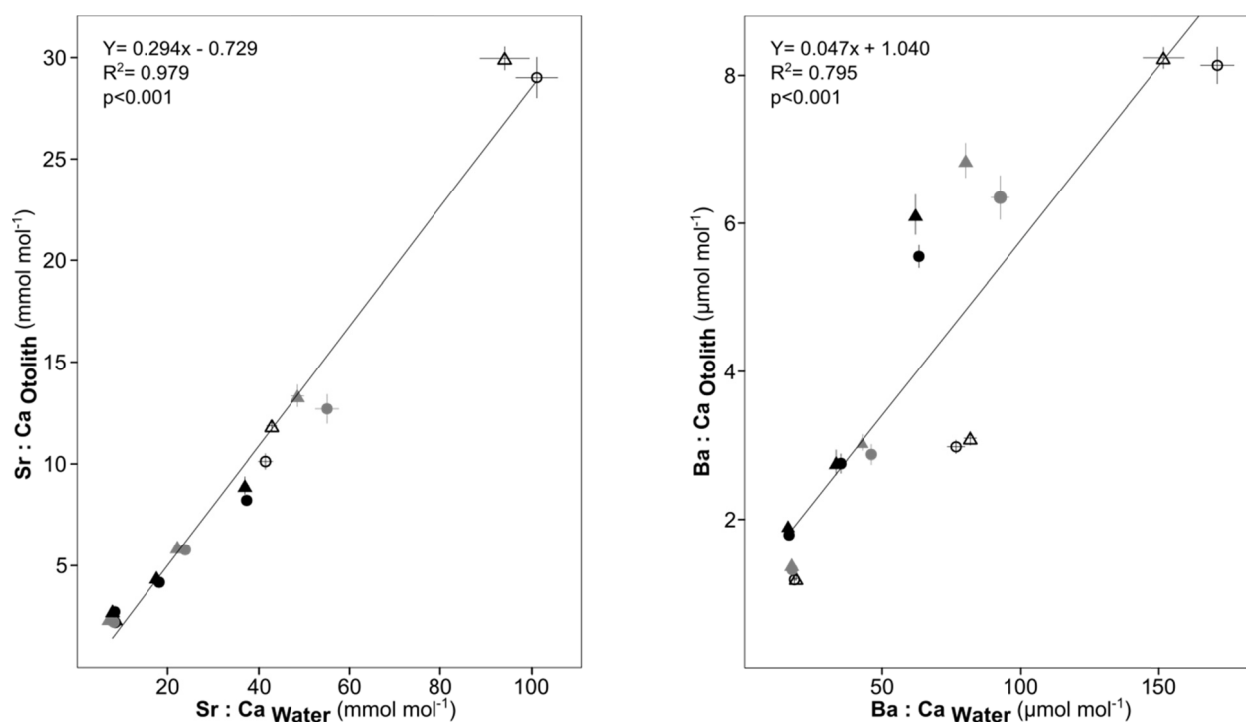


**Figure 2.** Mean and standard error bars of Ba:Ca<sub>Water</sub>, Ba:Ca<sub>Otolith</sub>, and  $D_{Ba}$  across concentration (Low, Medium, High), salinity (10, 20, 30) and temperature (21 °C - open circles, 25 °C - open triangles) experimental treatments of *Dicentrarchus labrax*.

**Table 4.** Results of permutational analysis of variance (PERMANOVA) examining the effects of salinity (S), temperature (T) and concentration (C) on the partition coefficient of strontium ( $D_{Sr}$ ) and barium ( $D_{Ba}$ ) in *Dicentrarchus labrax*. MS of 0.000 indicates values that are less than 0.0005.

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

		D <sub>Sr</sub>	D <sub>Ba</sub>
Source of variation	df	MS	MS
D <sub>Me</sub>			
Salinity	2	0.003	0.038***
Temperature	1	0.036**	0.003***
Concentration	2	0.038***	0.012***
Salinity x Temperature	2	0.000	0.000
Salinity x Concentration	4	0.026***	0.001***
Temperature x Concentration	2	0.000	0.000
Salinity x Temperature x Concentration	4	0.000	0.000
Tank (S x T x C)	36	0.002***	0.000**
Residual	216	0.001	0.000



**Figure 3.** Mean and standard error bars of  $Sr:Ca_{Otolith}$  plotted against  $Sr:Ca_{Water}$  and  $Ba:Ca_{Otolith}$  plotted against  $Ba:Ca_{Water}$  for experimental treatments of *Dicentrarchus labrax*. Linear regressions for each element and respective equations and regression coefficients ( $R^2$ ) are also shown. [Temperature: 21 °C (circles) and 25 °C (triangles). Salinity: 10 (open), 20 (grey) and 30 (black)].



#### 4. Discussion

Unravelling how the elemental composition of otoliths varies with the environment is required for determining fish movement and habitat use. The current experiment is an important contribution towards understanding the application of otolith Sr and Ba concentrations to reconstructions of fish life history.

Previous studies have highlighted the positive relationship between water chemistry and otolith composition in both freshwater and euryhaline or marine fish (e.g. Bath et al. 2000, Elsdon & Gillanders 2003, Kraus & Secor 2004, Miller 2009, Collingsworth et al. 2010). The current study is nevertheless, one of very few multifactor experiments that simultaneously addresses the influence of salinity, temperature and elemental water concentrations on otolith chemistry. Sr:Ca and Ba:Ca in juvenile *D. labrax* increased with increasing water elemental concentrations at all salinities. In other species, ambient water has been shown to be the primary contributor to otolith chemical composition for these elements (e.g. Walther & Thorrold 2006, Gibson-Reinemer et al. 2009, Webb et al. 2012). However, otolith elemental composition was not just a function of water elemental composition, as salinity and temperature were also influential. Moreover, elemental incorporation, based on partition coefficients ( $D_{Sr}$  and  $D_{Ba}$ ), were also influenced by environmental variables further highlighting the need to validate these effects for individual species and elements (Martin & Wuenschel 2006, Hicks et al. 2010, Miller 2011), prior to field applications.

The current experiment highlights the influence of combinations of environmental variables on otolith chemistry, and thus provides insight on their potential role and limitations for reconstructions of fish movements across finer environmental scales.  $Me:Ca_{Water}$  and  $Me:Ca_{Otolith}$  were positively related in both elements, yet a significant interactive effect of water elemental concentration and salinity on otolith elemental incorporation and composition was found. For Sr:Ca, the interaction resulted from enhanced elemental concentrations at lower salinity treatments attaining significantly greater  $Sr:Ca_{Otolith}$  in comparison to the other salinity treatments.  $Sr:Ca_{Otolith}$  concentrations were most likely driven by the Sr:Ca concentration in the rearing water ( $Sr:Ca_{Water}$ ), for which a similar interaction was also found. Kraus & Secor (2004) found conservative incorporation of strontium in otoliths across contrasting mixing curves, suggesting that the relationship between aqueous and otolith strontium is not strongly affected by salinity, or associated factors such as osmoregulation. Sr incorporation has been shown to be independent of salinity in abiotic aragonite (Gaetani & Cohen 2006) and there was no salinity effect on  $D_{Sr}$  as reported in other studies (Martin et al. 2004, de Vries et al. 2005, Phillis et al. 2011). Overall, a positive relation between Sr:Ca in otoliths and that in the water was found, covering a wide range of concentrations with no evidence of saturation, similar to Elsdon & Gillanders (2003). The positive effect of temperature on Sr:Ca was also substantiated with  $D_{Sr}$ , where increased Sr incorporation occurred at higher temperatures (see also Martin & Wuenschel 2006). Indeed, observed differences in  $D_{Sr}$  among temperature treatments were, in some cases, greater than those between salinities or concentration levels. Though, patterns of

$D_{Sr}$  varied within spiking treatments, an overall positive, linear relationship between otolith and water Sr:Ca was found. Ultimately, Sr:Ca has the potential to be applied as a tracer for habitat use as it reflected the underlying differences between environments that had distinct water elemental composition (Elsdon et al. 2008, Sturrock et al. 2012, Walther & Limburg 2012).

A positive relationship between water chemistry and otolith composition was also found for Ba:Ca, with an interactive effect between salinity and Ba:Ca water treatment concentrations. However, the resulting interactions were more complex, with a series of patterns arising among salinity and concentration level combinations. These may have the potential to complicate attempts to retrospectively identify individual fish movement across heterogeneous water masses such as estuaries [e.g. see Ba:Ca<sub>Otolith</sub> in Medium enhancement treatment; and also the variation of Ba:Ca in an estuarine hypersaline environment highlighted by Gillanders & Munro (2012)]. Similar multifaceted patterns of otolith Ba:Ca composition occurred for Chinook salmon *Oncorhynchus tshawytscha*, including a three-way interaction between temperature, salinity and water Ba:Ca ratios (Miller 2011). Such behaviour may be related to other physiological or iono-regulatory processes taking place (e.g. variability in Ba<sup>2+</sup> binding sites availability, uptake pathways) (Kalish 1989, 1991). Indeed facilitation or competition between trace elements, namely increased Ba:Ca incorporation at higher levels of Sr:Ca have been documented (de Vries et al. 2005, Collingsworth et al. 2010). Previous studies have also related  $D_{Ba}$  to ambient concentrations, discerning lower discrimination, thus higher uptake, at lower concentrations in the water, suggesting more Ba is proportionally incorporated at lower water concentrations (Bath et al. 2000, de Vries et al. 2005). Within each treatment, decreasing Ba:Ca<sub>Water</sub> along the salinity gradient resulted in higher elemental incorporation.

Temperature and salinity did not interact to affect otolith composition in *D. labrax* (as for other species, e.g. Chesney et al. 1998, Martin et al. 2004, Martin & Wuenschel 2006). A temperature x salinity interaction was found by Elsdon & Gillanders (2002) when only these two factors were investigated; but when elemental concentration was also included as a factor, in a subsequent salinity, temperature and concentration level experiment conducted on the same species, no salinity x temperature interaction was found (Elsdon & Gillanders 2004). Differences in results were attributed to changes in the water chemistry rather than salinity itself. Similarly, Miller (2011) argues that the salinity x temperature interaction found for Sr in fact reflects a concentration x temperature interaction, in agreement with salinity independent Sr incorporation (Kraus and Secor 2004, Gaetani & Cohen 2006).

Many laboratory experiments on otolith elemental composition have been carried out. Yet, general patterns are often hard to find; and in part this may be due to species-specific differences (e.g. Reis-Santos et al. 2008, Melancon et al. 2009) whereby differences in metabolic rates or physiological processes may influence elemental incorporation into otoliths (Kalish 1989, 1991). For instance, for temperature, independent of its relationship to growth rate, most studies have, as in the current one, found a positive relationship between temperature and Sr:Ca in otoliths however, negative and non-significant correlations between otolith Sr:Ca and temperature have also been reported (see reviews by Campana 1999, Elsdon

et al. 2008, Sturrock et al. 2012). Besides water chemistry, salinity or temperature, other factors are known to influence otolith chemistry. Therefore, in addition to the controlled chemical environment with stable temperature and salinity regimes, a standardised diet was fed to all fish to minimize any residual effects of food on otolith chemistry (Walther & Thorrold 2006, Webb et al. 2012), and *D. labrax* were all from the same brood stock and age, to minimize potential genetic (Clarke et al. 2011) or ontogenetic effects (Walther et al. 2010). We also used artificial seawater in an attempt to minimize variations in Sr and Ba fluctuations (e.g. Bath et al 2000). Yet, variability in otolith chemistry among fish in individual tanks was still found. These differences are likely due to variations in rearing conditions throughout the experiment (even if no water tank effects were found), namely among enhanced concentration treatments where larger error values were observed; as well as to variability in individual's physiological and iono-regulatory responses to experimental conditions, which affect ion transfer / transport across membranes and consequently otolith elemental incorporation (Kalish 1989, 1991, Varsamos et al 2001, Melancon 2009). Further investigations on physiological and genetic mechanisms affecting otolith elemental incorporation are clearly needed.

Ultimately, otolith elemental composition was a function of environmental factors, supporting the assumption that variations in the environment are reflected in otolith composition. In field trials, relationships between water and otolith chemistry have also been observed. For instance, the relationship between ambient and otolith Ba has been coupled with its natural patterns of variation, enabling Ba to be applied as tracer to reconstruct fish life histories as it occurs in greater abundance in freshwater compared to seawater (Elsdon & Gillanders 2005a, Hamer et al. 2006). However, beyond assessing movement from seawater to freshwater, untangling movement and habitat use patterns at finer scales still pose a greater challenge. Macdonald & Crook (2010) illustrated the difficulties faced in resolving otolith elemental profiles of wild caught euryhaline fish for environmental reconstruction. The lack of a predictive and mechanistic understanding of the generality of ion uptake to otoliths still limits our extrapolation from laboratory to field collections (Elsdon & Gillanders 2005b, Dorval et al 2007). Results from the current study detailing how elemental factors control otolith chemistry are a step towards reconstructing environmental histories in dynamic environments, across gradients of temperature, salinity and ambient elemental concentration. This could be valuable to determine differential juvenile nursery habitat use patterns and identify essential habitats for marine migrant fish such as *D. labrax*. Yet, laboratory validation studies are only a starting point for accurate habitat reconstructions as there is scope for discrepancies between field and laboratory assessments that must also be evaluated, particularly in dynamic environments such as estuaries (Kraus & Secor 2004, Elsdon & Gillanders 2005b, Dorval et al 2007). Combining laboratory trials and species specific information on otolith elemental incorporation, with comprehensive knowledge of expected ranges of variation within the natural environment of ambient water concentrations and environmental variables will enable us to determine realistic ensuing thresholds of otolith elemental composition (Hicks et al. 2010, Macdonald & Crook 2010). Moreover, the inclusion of additional markers, such as otolith  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  (Kerr et al.

2007, Dorval et al. 2011, Limburg et al. 2011) as records of environmental conditions (e.g. salinity, temperature, hypoxia), may further aid the interpretation of fish movement. Ultimately, accurately resolving fish habitat use, movement and migration patterns is essential to the development of effective management and conservation plans.

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## CHAPTER 4

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### **Temporal variability in estuarine otolith elemental fingerprints: implications for connectivity assessments**

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## Temporal variability in estuarine otolith elemental fingerprints: implications for connectivity assessments

**Abstract:** Chemical composition of fish otoliths can provide valuable information for determining the nursery value of estuaries to adult populations of coastal fishes. However, understanding temporal variation in elemental fingerprints at different scales is critical as it can potentially confound spatial discrimination among estuaries. Otolith elemental ratios (Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca and Pb:Ca) of *Platichthys flesus* and *Dicentrarchus labrax*, from multiple estuaries along the Portuguese coast in two years and three seasons (spring, summer and autumn) within a year, were determined via Laser Ablation Inductively Coupled Plasma Mass Spectrometry. Elemental fingerprints varied significantly among years and seasons within a year but accurate classifications of juvenile fish to estuarine nursery of origin were attained (77 % to 96 % overall cross-validated accuracy). Although elemental fingerprints were year specific, variation among seasons did not hinder spatial discrimination. Estuarine fingerprints of pooled seasonal data were representative of the entire juvenile year class and attained high discrimination (77 % and 80 % overall cross-validated accuracy for flounder and sea bass, respectively). Incorporating seasonal variation resulted in up to an 11 % increase in correct classification of individual estuaries, in comparison to seasons where accuracies were lowest. Overall, understanding the implications of temporal variations in otolith chemistry for spatial discrimination is key to establish baseline data for connectivity studies.

**Keywords:** Otolith chemistry; temporal variation; connectivity; estuaries; nurseries; Portugal

### 1. Introduction

A conspicuous feature in estuarine systems is the high densities of juvenile fish, including species for which adults live in adjacent marine areas. Many of these species rely on estuaries and associated habitats as nursery grounds, where juveniles remain for months to years before recruiting to adult populations in coastal areas. As habitats used by juveniles and adults are spatially segregated it is fundamental to determine the movement between these sub-populations, i.e. connectivity, and estimate the fraction of recruits that originate from different nursery areas (see Beck et al. 2001, Able 2005, Vasconcelos et al. 2011) in order to address population dynamics, colonization patterns and resilience to harvesting (Thorrold et al. 2001). Moreover, knowledge of this link is fundamental to identify ecologically important habitats and underpins sustainable management and conservation efforts (Cowen et al. 2006).

Contrary to the initial belief that fish populations are predominantly open, recent evidence challenges this paradigm of broad dispersal (Cowen et al. 2000, Swearer et al. 2002). Nevertheless, quantifying movement of marine organisms and unravelling the links between estuarine juvenile and coastal adult populations is a complex task. Despite considerable progress, juvenile fish are inherently difficult to mark using conventional tags but recent technological advances enable the interpretation of distinct biological features as natural tags. These have become commonly used and chemical composition of otoliths has developed into one of the leading techniques to assess fish movement (Gillanders 2009).

The use of chemical composition of otoliths as natural tags is possible because otoliths are

metabolically inert, grow continuously and incorporate trace elements as they accrete (reviewed in Campana 1999, Elsdon et al. 2008). Hence, as otoliths form, an elemental fingerprint is recorded and due to the intrinsic relation between otolith growth and fish age the environmental record of an individual at specific time periods can be determined. Reconstructing migratory patterns (Tsukamoto & Arai 2001, Jessop et al. 2002, Daverat et al. 2011), discriminating natal or nursery origins (Rooker et al. 2001, Brown 2006, Vasconcelos et al. 2007, Bradbury et al. 2011) and assessing differential contributions of juvenile areas to adult populations (Thorrold et al. 2001, Gillanders 2005, Vasconcelos et al. 2008, Chittaro et al. 2009), have all been accomplished with otolith chemistry.

A vital requirement to the successful application of otolith elemental fingerprints is the occurrence of spatial variation at relevant scales. If elemental fingerprints are found to be site specific, namely at pertinent management scales such as estuaries, then their nursery value can be ascertained. However, assessing temporal variation in elemental tags is also critical. Lack of temporal stability has the potential to confound spatial discrimination and promote data misinterpretation. Although it is likely that elemental fingerprints in estuaries vary amongst years, or even shorter time periods, due to changes in environmental conditions, with implications for the retrospective determination of nursery estuaries of adult fish, the subject of temporal variability has seldom been addressed in different coastal environments (but see Gillanders 2002, Hamer et al. 2003, Swearer et al. 2003, Clarke et al. 2009, Mateo et al. 2010).

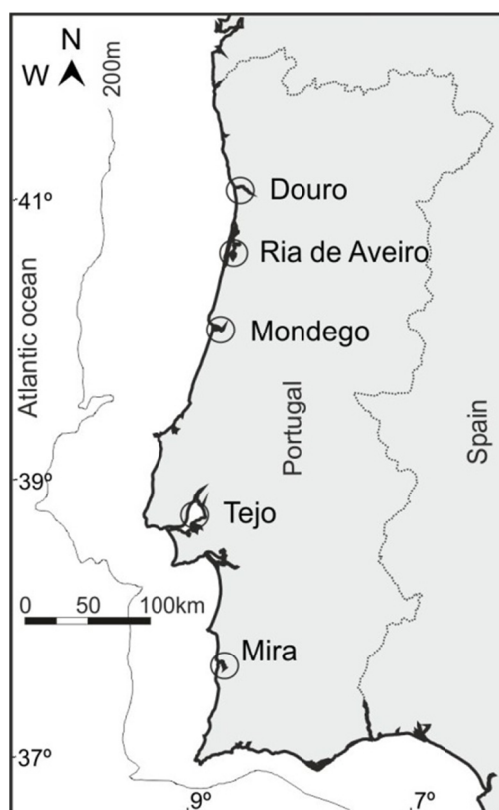
Along the Portuguese coast several estuaries are recognized for their importance for juvenile flounder *Platichthys flesus* (Linnaeus 1758) and European sea bass *Dicentrarchus labrax* (Linnaeus 1758). These marine migrant species are valuable resources in Portuguese fisheries and their use and dependence on estuarine areas as juveniles along this coast well documented (Cabral et al. 2007, Martinho et al. 2008, Vasconcelos et al. 2010). The suitability of otolith elemental fingerprints to discriminate among estuarine nurseries and to provide evidence of connectivity with adult populations of fishes along the Portuguese coast has been previously highlighted (Vasconcelos et al. 2007, Vasconcelos et al. 2008). However, the temporal variability in these natural tags, at both annual and seasonal time scales, has not been addressed and therefore its repercussions for spatial discrimination of estuarine nurseries and the accurate identification of important juvenile nursery estuaries for fish are unknown.

This study evaluated the temporal variation in elemental fingerprints from otoliths of juvenile *P. flesus* and *D. labrax* in estuaries of the Portuguese coast and whether these natural tags are representative of estuarine nurseries through time. Specific aims were to 1) assess short term (among seasons) and long term variation (between years) in estuarine elemental fingerprints; 2) examine the implications of seasonal variation in the accuracy of spatial discrimination among sampling periods, including how it affects the characterization of year specific elemental fingerprints; and 3) explore overlap, if any, in elemental fingerprints from estuaries and years that could hinder their use in the retrospective identification of adult origins. This is a key step to avoid inadequate interpretations of otolith chemical data whilst simultaneously establishing baseline fingerprints of potential source habitats for connectivity assessments.

## 2. Material and methods

### 2.1. Fish Collection

Juvenile age-0 flounder *Platichthys flesus* and sea bass *Dicentrarchus labrax* were collected with a beam trawl in estuarine systems along the Portuguese coast (Figure 1). Selected estuaries are key for juveniles of these species (Cabral et al. 2007, Martinho et al. 2008, Vasconcelos et al. 2008, Vasconcelos et al. 2011) and sampling took place in July (summer) 2006; and also in May, July and October (spring, summer and autumn, respectively) 2009, following the start of estuarine settlement by both species. Sampling occurred when elevated densities of age-0 fish are found within these estuarine nursery sites (typically between early spring and late autumn) (Cabral & Costa 2001, Dolbeth et al. 2008, Martinho et al. 2008, Vasconcelos et al. 2010), and fish collections were carried out in the shortest time frame possible within each month. Age-0 juveniles can show elevated nursery fidelity (Vasconcelos et al. 2010, Vinagre et al. 2010) and in some estuaries juveniles occur in high densities in sites separated by a few kilometres. Therefore, individuals were collected from these multiple sites and pooled by estuary to account for any within estuary variation. Upon collection, fish were transported on ice and frozen at the laboratory. Water temperature (°C) and salinity were measured at each collection site and sampling time using a portable multiparameter instrument (YSI 6820) (Table 1).



**Figure 1.** Sampled estuaries along the Portuguese coast in 2006 and 2009.

**Table 1.** Mean (and standard error) temperature (°C) and salinity of sampled estuaries in 2006 and 2009

Estuary	Temperature				Salinity			
	2006		2009		2006		2009	
	July	May	July	October	July	May	July	October
Douro	24.1 (0.1)	18.3 (0.1)	23.7 (0.2)	23.7 (0.2)	23.7 (0.2)	23.7 (0.2)	2.4 (0.5)	2.2 (0.4)
Ria de Aveiro	24.9 (0.2)	16.7 (0.2)	24.9 (0.3)	24.9 (0.3)	24.9 (0.3)	24.9 (0.3)	25.7 (1.3)	10.1 (1.0)
Mondego	23.4 (0.2)	19.1 (0.1)	24.2 (0.2)	24.2 (0.2)	24.2 (0.2)	24.2 (0.2)	13.3 (1.5)	5.4 (1.1)
Tejo	25.5 (0.1)		21.5 (0.3)	21.5 (0.3)	21.5 (0.3)		18.6 (1.1)	5.1 (0.0)
Mira	26.9 (0.1)		25.7 (0.1)	25.7 (0.1)	25.7 (0.1)		21.5 (0.9)	27.4 (0.3)

## 2.2 Otolith preparation

Total fish length was measured (to the nearest mm) (Table 2) and sagittal otoliths extracted, using plastic forceps, washed and cleaned of adhering tissue with ultra-pure water and allowed to air dry in microcentrifuge tubes in a positive pressure laminar flow fume hood. All plastic and glassware used in otolith preparation was previously acid washed in 10 % p.a. nitric acid (HNO<sub>3</sub>) (see Vasconcelos et al. 2007 for more details). The right otolith of each fish was embedded in epofix resin (Struers) within latex moulds and placed in an oven at 54 °C to harden. The resin was previously spiked with 30 ppm indium (In) to act as a marker for the resin during analysis. Otoliths were sectioned transversely through the nucleus using a Buehler Isomet low speed saw equipped with twin diamond edge blades and spacers. Resulting otolith sections were polished to c. 250 µm with lapping film (30 µm, 9 µm and 3 µm successively, using ultrapure water to remove surface contamination between each stage) and mounted on glass slides with thermoplastic glue (CrystalBond 509), also spiked with 30 ppm In. Slides were then cleaned, sonicated and triple-rinsed with ultrapure water, dried under a laminar flow hood and stored individually in sealed plastic bags.

## 2.3. Sample analysis

Chemical composition of otoliths was determined with a New Wave 213 nm UV high performance (Nd:YAG) laser microprobe coupled to an Agilent 7500cs inductively coupled plasma mass spectrometer (ICP-MS). The laser operating conditions were adapted from Munro et al. (2008), with repetition rate of 5 Hz and c. 10 J cm<sup>-2</sup>. Laser ablations occurred inside a sealed chamber with resultant material and sample gas transported to the ICP-MS via a smoothing manifold in an argon and helium stream. Sectioned otoliths were analysed using a 30µm spot on the outside edge of the otoliths to quantify recent elemental incorporation, i.e. material laid down shortly before capture that is assumed to be representative of the site and time of capture. A similar region of each otolith was ablated.

**Table 2.** Mean (and standard error) fish total length in mm (Lt) and sample size (n) of *Platichthys flesus* and *Dicentrarchus labrax* from each estuary and sampling period

Species	Estuary	2006		2009					
		July		May		July		October	
		Lt	n	Lt	n	Lt	n	Lt	n
<i>Platichthys flesus</i>									
	Douro	60 (2)	20	42 (3)	17	82 (2)	20	98 (4)	15
	Ria de Aveiro	82 (3)	11	-	-	74 (4)	15	-	-
	Mondego	67 (3)	22	47 (1)	20	66 (3)	20	86 (3)	20
<i>Dicentrarchus labrax</i>									
	Douro	69 (2)	25	-	-	53 (6)	5	114 (3)	18
	Ria de Aveiro	51 (5)	15	-	-	66 (2)	20	135 (1)	21
	Mondego	51 (2)	20	-	-	62 (2)	25	94 (3)	25
	Tejo	80 (3)	20	-	-	58 (2)	25	109 (3)	25
	Mira	70 (2)	22	-	-	90 (3)	19	123 (1)	9

Seven elements were quantified ( $^7\text{Li}$ ,  $^{24}\text{Mg}$ ,  $^{55}\text{Mn}$ ,  $^{65}\text{Cu}$ ,  $^{88}\text{Sr}$ ,  $^{138}\text{Ba}$  and  $^{208}\text{Pb}$ ), along with  $^{43}\text{Ca}$  which was used as the internal standard (Yoshinaga et al. 2000). These elements have been successfully applied to discriminate among these estuaries and species (Vasconcelos et al. 2007, Reis-Santos et al. 2008, Tanner et al. 2011).  $^{115}\text{In}$  was also measured but solely as a marker to discriminate between spiked resin or CrystalBond and otolith matrices.

Otoliths were analysed in several sampling sessions; and within each session were analysed in random order. Prior to each ablation, background concentrations within the chamber were measured for correction of individual sample concentrations; and the chamber was purged after each ablation to remove gas from previous ablations (Lahaye et al. 1997). Certified glass NIST 612 (National Institute of Standards and Technology) was used as calibration standard. External precision (% relative standard deviation) was assessed via repeated measurements of another certified reference material (MACS-3, United States Geological Survey) ( $n = 64$ ) and were as follows: 3.4 % (Li), 4.8 % (Mg), 5.4 % (Mn), 7.3 % (Cu), 2.1 % (Sr), 3.2 % (Ba) and 9.0 % (Pb). Standards were analysed at start and end of each session and after every 10 otolith ablations. Data reduction, including background corrections, mass count data conversion to concentrations (ppm) and limits of detection (LOD) were performed for each individual sample via Glitter software (GEMOC, Macquarie University, Sydney Australia). LOD (in  $\mu\text{g g}^{-1}$ ) were 0.06 (Li); 0.32 (Mg); 0.11 (Mn); 0.15 (Cu); 0.03 (Sr); 0.02 (Ba); 0.01 (Pb). Otolith data were consistently above detection limits, and all acquired data were kept for statistical analyses. For all individual elements, at least 94 % of data were above LOD, including 100 % for Mg, Sr and Ba. The few samples below LOD did not affect a particular estuary or species. Overall, differences among sampled groups will be valid irrespective of these few data below LOD (Ben-Tzvi et al. 2007, Fairclough et al. 2011, Hamer et al. 2012). There are no strict protocols to treat data below instrument LOD. In fact, it has been argued that data are unnecessarily discarded when below LOD, as these data may be useful in discriminating groups (Ben-Tzvi et al. 2007).

## 2.4. Data analysis

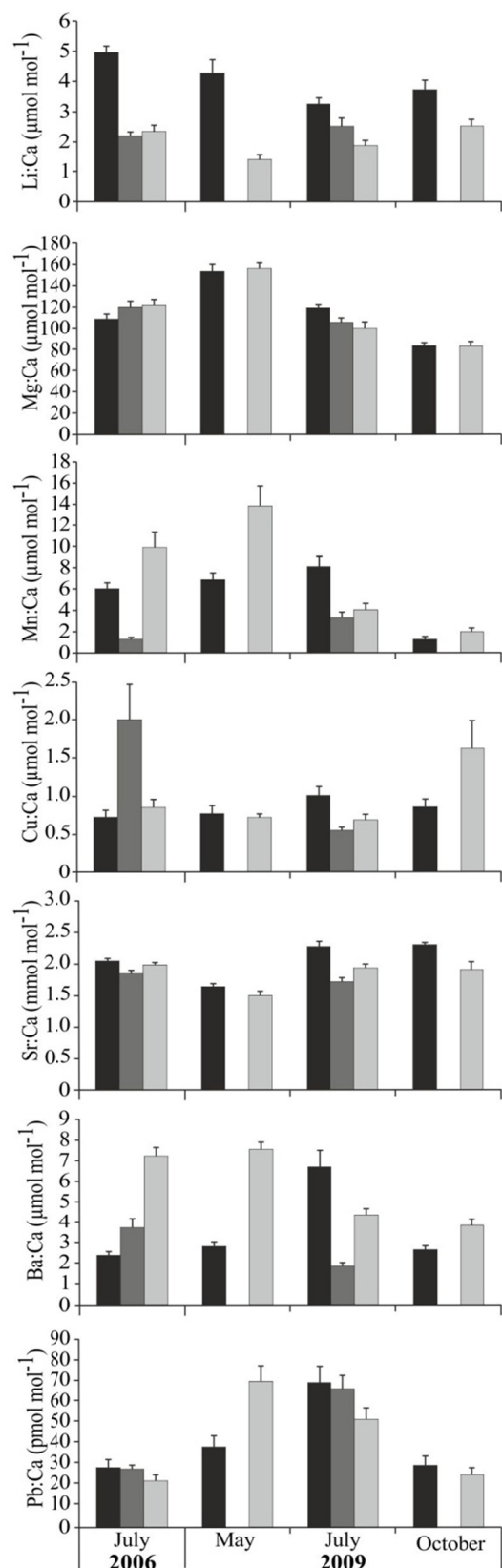
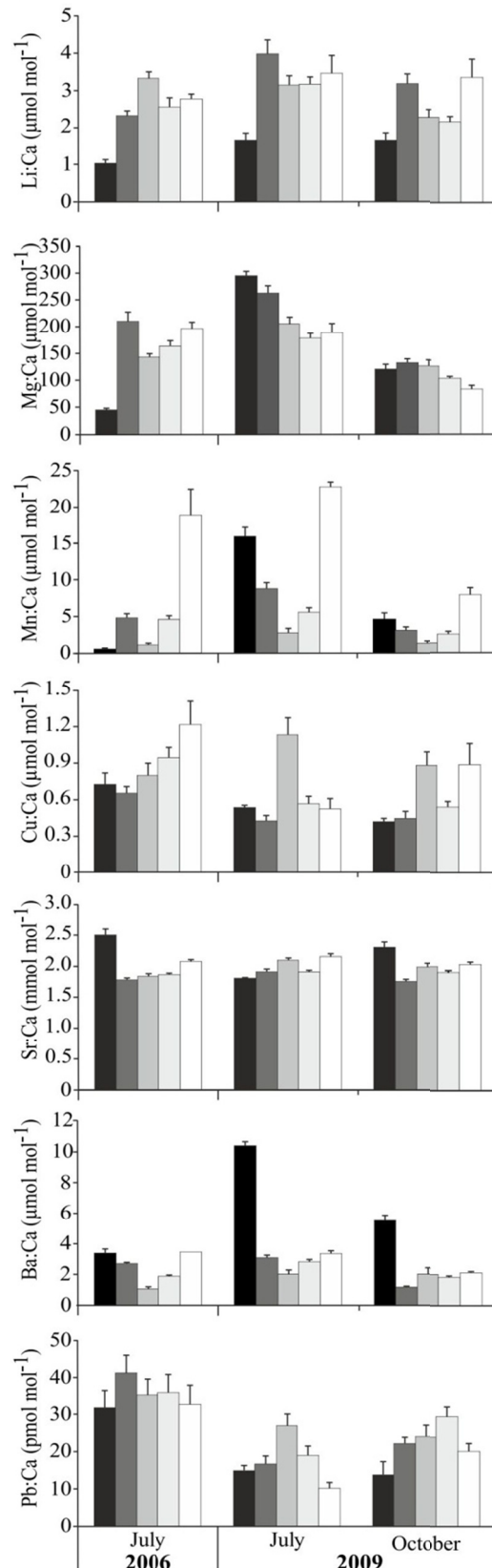
Otolith elemental concentration data were converted to molar concentrations and standardised to calcium. All data analyses were carried out on the element:Ca data.

Comparisons of the otolith elemental fingerprints were conducted using permutational multivariate analysis of variance (PERMANOVA), with estuaries and years or months as factors. Species were analysed independently. Variables were  $\log(x + 1)$  transformed and PERMANOVA was based on Euclidean distance dissimilarity matrices. When significant differences were found, *a posteriori* pairwise comparisons among all levels of a factor were also examined. Variation in otolith elemental fingerprints between years was assessed considering all estuaries, and within year variability was assessed among estuaries sampled in at least two months within 2009. Despite extensive sampling, age-0 juveniles of *P. flesus* were not found in the Ria de Aveiro in May and October 2006. In May 2009, *D. labrax* were only found in the Tejo estuary, therefore this sampling season was disregarded for this species. Canonical variate plots of 95 % confidence limits around the mean of the data for each group were also used to display temporal and spatial variation of the multi-elemental tags in reduced space.

To assess the ability of estuarine elemental fingerprints to successfully classify juvenile fish to their estuary of origin and how this changed between years, and among months within a year, Canonical Analysis of Principal Coordinates (CAP) was used. CAP is a constrained ordination for discriminating among *a priori* groups, and the leave-one-out approach provides a reasonable and unbiased measure of how distinct groups are in multivariate space (Anderson & Willis 2003). Cross-validated classification accuracies were analysed for each year and each month within a year. CAP analyses were run with the elemental data pooled from all sampling seasons within a year to determine how variation among seasons may affect the definition of an elemental fingerprint for a specific recruitment year. To ascertain the effect of inter-annual variation on estuarine discrimination, CAP analyses were also run with data from both years pooled. Furthermore, otolith elemental fingerprints of fish collected in one year were examined to test if they could be used as a reliable proxy to classify fish from another year. This was verified using the CAP analysis of a year and adding the samples from the other year as unknowns. For all CAP and PERMANOVA tests 4999 unrestricted random permutations of the raw data were used.

## 3. Results

Otolith elemental fingerprints differed among estuaries and temporal scales, and the concentration of individual elements also varied significantly. Overall, some general patterns in the element:Ca ratios could be observed (Figure 2). For *P. flesus*, Li:Ca ratios from the Douro were always highest independent of year or sampling period but in contrast they were always the lowest for *D. labrax* within the same estuary. Conspicuous peaks in Mn:Ca were evident in the otolith elemental composition of fish collected in the Mira estuary. A decreasing trend in element:Ca ratios over sampling seasons within 2009 was observed for Mg:Ca and Mn:Ca, as well as Ba:Ca for *D. labrax*, in all estuaries.

A) *Platichthys flesus*B) *Dicentrarchus labrax*

**Figure 2.** Mean (and standard error) element:Ca ratios (Li, Mg, Mn, Cu, Sr, Ba, Pb) in otoliths of juvenile age-0 (A) *Platichthys flesus* and (B) *Dicentrarchus labrax* collected in July 2006 and May, July and October 2009 within estuaries along the Portuguese coast: Douro (■), Ria de Aveiro (■), Mondego (■), Tejo (■), and Mira (□).

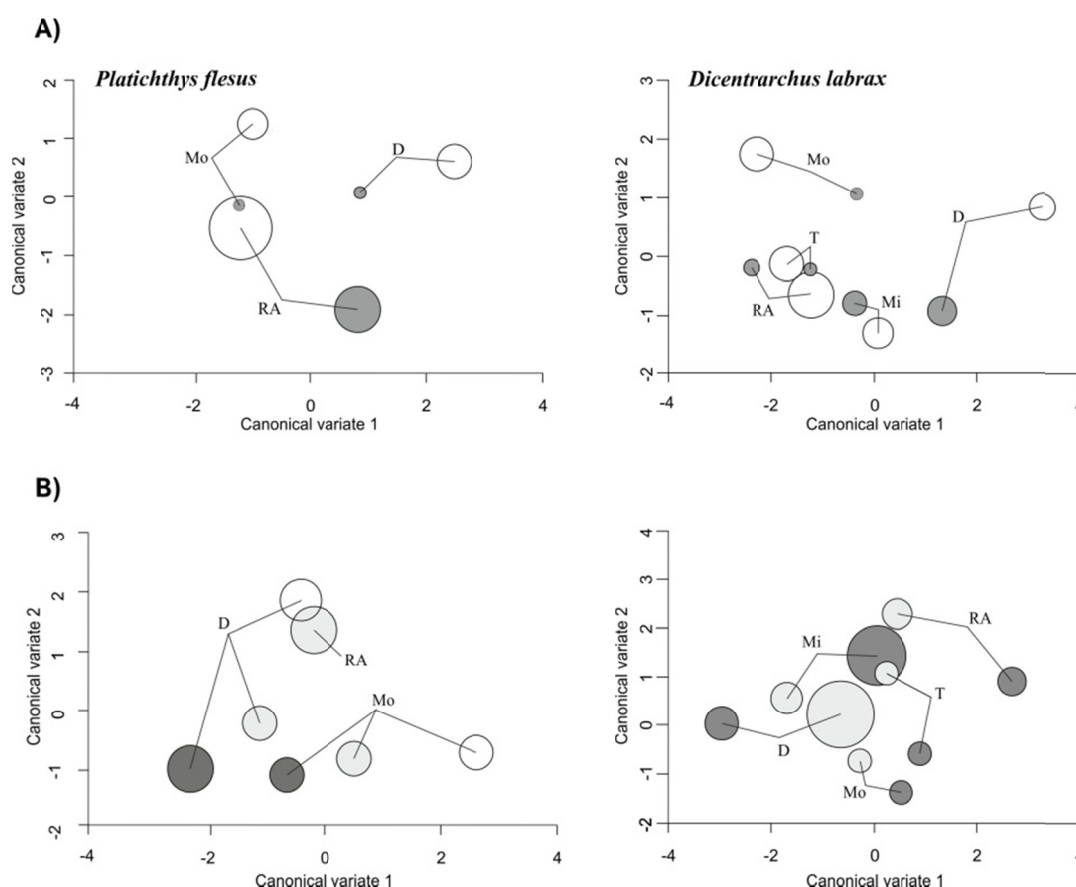


Comparison of the multi-element estuarine otolith fingerprints between years revealed significant differences for each species, including estuary and year interactions (Table 3). Pairwise post hoc tests showed significant differences for all pairs of levels within each factor. Univariate results of PERMANOVA revealed significant differences in individual element:Ca ratios for either year and/or estuary, with the exception of Mg:Ca for *P. flesus* (Table 3). Notably, Ba:Ca was significantly different in all cases for both species. The 95 % confidence limits around the mean of the elemental fingerprints in the canonical variate plots also highlighted the inter-annual differences in each estuary (Figure 3A). Differences between years were evident for all estuaries, with elemental fingerprints of *D. labrax* from the Tejo estuary from both years closer to each other. Even though overall spatial discrimination in fingerprints was observed, the Tejo and Ria de Aveiro estuaries for *D. labrax* were clustered closer together in the canonical space; and some overlap occurred between Ria de Aveiro 2006 and Tejo 2009 for this species, and Ria de Aveiro 2006 and Mondego 2009 for *P. flesus*.

**Table 3.** Multivariate and univariate results of PERMANOVA examining temporal variation in otolith element:Ca ratios (a) between years and (b) within year for *Platichthys flesus* and *Dicentrarchus labrax*. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001

Species	df	MS							
		All elements	Li:Ca	Mg:Ca	Mn:Ca	Cu:Ca	Sr:Ca	Ba:Ca	Pb:Ca
<i>Platichthys flesus</i>									
a) Between years (2006 & 2009)									
Estuary	2	20.71***	4.96***	0.01	6.92***	0.18	0.10***	6.36***	1.55*
Year	1	17.38***	0.39*	0.07	0.04	0.66**	0.06	0.67*	13.72***
Estuary x year	2	8.75***	0.22	0.15	2.43**	0.95***	0.03	2.14***	0.32
Residuals	174	1.49	0.09	0.07	0.51	0.10	0.02	0.13	0.43
b) Within year (2009)									
Estuary	2	9.70***	3.50***	0.05	1.25***	0.12	0.25***	4.15***	9.70***
Month	2	31.79***	0.32*	3.45***	19.03***	0.34*	0.41***	1.45***	31.80***
Estuary x month	2	9.49***	0.56**	0.11*	3.52***	0.36*	0.02	2.83***	9.49***
Residuals	120	0.98	0.08	0.03	0.22	0.09	0.01	0.09	0.98
<i>Dicentrarchus labrax</i>									
a) Between years (2006 & 2009)									
Estuary	4	45.17***	4.96***	5.19***	27.78***	0.35***	0.21***	5.63***	2.76***
Year	1	28.39***	0.39*	0.97**	7.34***	1.17***	0.01	0.88**	17.47***
Estuary x year	4	12.22***	0.22	4.18***	5.36***	0.27**	0.04***	1.19***	0.40
Residuals	284	1.25	0.09	0.13	0.34	0.05	0.01	0.09	0.45
b) Within year (2009)									
Estuary	4	23.60***	0.89***	1.01***	14.91***	0.62***	0.04***	4.36***	23.59***
Month	1	55.85***	0.71*	16.92** *	30.32***	0.01	0.01	5.35***	55.85***
Estuary x month	4	3.56***	0.13	0.23*	1.18***	0.11*	0.03***	0.46***	3.56***
Residuals	182	0.96	0.12	0.08	0.24	0.04	0.01	0.08	0.96

Seasonal variation in both species' elemental fingerprints was also marked. Multi-element comparisons with PERMANOVA were significant for all analysed factors (estuary and month) as well as for most of the individual elements (Table 3). All pairs of levels in each factor were also significantly different based on post hoc pairwise comparisons. Some of the element:Ca ratios, such as Mn:Ca, Ba:Ca and Pb:Ca, showed a significant interaction between estuaries and months for both *P. flesus* and *D. labrax* (Table 3). For each estuary, the elemental fingerprints represented in the canonical space differed for each sampling season (Figure 3B). Yet, some overlap occurred between the elemental fingerprints of *P. flesus* from the Ria de Aveiro collected in July and from the Douro in May. For *D. labrax*, the elemental fingerprints from the Mira in October overlapped those of the Tejo in July.



**Figure 3.** Canonical variate plots summarising variation in otolith elemental fingerprints in juvenile age-0 *Platichthys flesus* and *Dicentrarchus labrax* among estuaries along the Portuguese coast in: A) fish collected in 2006 (open) and 2009 (dark grey) and B) fish collected in May (open), July (grey) and October (dark grey) 2009. Ellipses represent 95 % confidence limits for the mean. [estuary abbreviations - Douro (D), Ria de Aveiro (RA), Mondego (Mo), Tejo (T), Mira (Mi)].

Results of the CAP analysis revealed an overall high accuracy of classification of juvenile fish to their estuaries of origin. Cross-validated classification results varied between years as well as between species (Table 4). For *P. flesus*, classification accuracies to individual estuaries within all sampling seasons and years ranged from 70.0 % to 100 %. For juvenile flounder collected in 2006, the overall correct classification was 96.3 %. In general, in 2009 spatial discrimination among estuaries decreased, with overall correct classification between 71.4 %

and 89.2 %. When seasonal data for 2009 were pooled, *P. flesus* correct classification ranged from 73.3 % (Mondego) to 80.8 % (Douro), with a total of 77.2 % of individuals correctly assigned. For *D. labrax*, classification to individual estuaries in 2006 ranged from 85.0 %, for the Tejo, up to 100 %, for the Douro estuary, with overall 92.2 % correct classifications. In general, classification accuracies also decreased in 2009 for this species. Assessing July and October individually, there were a total of 81.9 % and 72.4 % correctly classified individuals, respectively: in July, correct classifications per estuary were between 65.0 % (Ria de Aveiro) and 100.0 % (Douro and Mira) and for October between 64.0 % (Tejo) and 88.9 % (Douro and Mira).

**Table 4.** Cross-validated correctly classified individuals (%) of juvenile *Platichthys flesus* and *Dicentrarchus labrax* to their estuary of origin in each sampling period. Classification to estuary of origin was based on otolith elemental signatures and canonical analysis of principal components. Values in bold indicate classifications with May, July and October 2009 pooled. Sample size is also shown (n)

Species		Cross-validated correctly classified individuals (%)						
Year	Month	n						
<i>Platichthys flesus</i>								
		Douro	Ria de Aveiro	Mondego	Overall			
2006	July	100	100	90.9	96.3	53		
2009	May	88.2		90.0	89.2	37		
	July	80.0	86.7	70.0	74.5	55		
	October	86.7		70.0	71.4	35		
	Pooled	80.8	80.0	73.3	77.2	127		
<i>Dicentrarchus labrax</i>								
		Douro	Ria de Aveiro	Mondego	Tejo	Mira	Overall	n
2006	July	100	93.3	95.0	85.0	86.4	92.2	102
2009	July	100	65.0	80.0	80.0	100	81.9	94
	October	88.9	71.4	68.0	64.0	88.9	72.4	98
	Pooled	100	73.2	74.0	74.0	96.4	80.2	192

When fingerprints incorporating all 2009 sampling seasons were used to characterize the year class, correct classifications averaged 80.2 %, with classification accuracy of individual estuaries between 73.2 % (Ria de Aveiro) and 100 % (Douro). Overall, incorporating seasonal variation in elemental fingerprints resulted in up to an 11 % increase in classification of individual estuaries and c. 6 % or 8 % rise in overall classification of *P. flesus* and *D. labrax*, respectively, in comparison to the sampling periods where accuracies were lower. Although pooling data from the 2006 and 2009 years together to form a single multi-year elemental tag for each estuary rendered an acceptable overall correct classification of 70.5 % for *P. flesus* and 71.8 % for *D. labrax*, these results were lower than for the individual years and classification was significantly reduced for individual estuaries (Table 5). For example, in *D. labrax* correct

classifications for both the Ria de Aveiro and Tejo estuaries did not surpass 57.1 %. Furthermore, classification schemes using elemental tags representative of one recruitment year as a proxy to classify the other (treated as of unknown origin) failed to produce adequate levels of correct classifications, suggesting that the elemental signatures were specific to individual years. For *P. flesus*, using the 2006 year class as a proxy for 2009 and vice-versa, overall classification accuracies were only 57.5 % and 54.7 %, respectively. Similar classification accuracy (56.9 %) was attained when using the CAP functions of 2009 *D. labrax* juveniles to classify 2006 fish; which decreased further to 13.0 % when using the 2006 elemental fingerprints as proxies to classify 2009 sea bass.

**Table 5.** Cross-validated correctly classified individuals (%) of juvenile *Platichthys flesus* and *Dicentrarchus labrax* to their estuary of origin pooling all samples for 2006 and 2009. Classification to estuary of origin was based on otolith elemental signatures and canonical analysis of principal components. Also shown, classification accuracy to estuary of origin using otolith elemental fingerprints of one year as proxy to classify individuals from the other (treated as of unknown origin). Data for 2009 includes fish collected in May, July and October. Sample size is also shown (n)

Species	Cross-validated correctly classified individuals (%)						
<i>Platichthys flesus</i>							
	Douro	Ria de Aveiro	Mondego	Overall			n
2006 & 2009	75.0	65.4	68.3	70.5			180
Proxies							
2006 for 2009	59.6	53.3	56.7	57.5			127
2009 for 2006	90.0	18.8	40.9	54.5			53
<i>Dicentrarchus labrax</i>							
	Douro	Ria de Aveiro	Mondego	Tejo	Mira	Overall	
2006 & 2009	91.7	57.1	72.8	57.1	80.0	71.8	294
Proxies							
2006 for 2009	13.0	2.4	0.0	0.0	75.0	13.0	192
2009 for 2006	100	33.3	45.0	45.0	45.5	56.9	102

#### 4. Discussion

Otolith elemental concentrations in juvenile *P. flesus* and *D. labrax* were within the range reported in other studies (e.g. Clarke et al. 2009, Leahey et al. 2009, Cuveliers et al. 2010, Tanner et al. 2011) and revealed significant variation among estuaries and temporal scales. The spatial variation in the multi-element fingerprints enabled accurate classification of juvenile fish to their estuarine nursery of origin with classification accuracies varying between years as well as within year. Elemental fingerprints were year specific. However, variation among seasons did not hinder spatial discrimination and estuarine fingerprints of pooled seasonal data were representative of the entire juvenile year class. Overall, incorporating seasonal variation in elemental fingerprints enhanced assignment accuracies, with classifications similar to other

studies in estuarine systems (Thorrold et al. 1998, Gillanders & Kingsford 2003, Vasconcelos et al. 2007), and will be important as baseline data for connectivity assessments.

Marine migrant species provide interesting frameworks in which to evaluate metapopulation dynamics and estimate connectivity between geographically segregated subpopulations (Thorrold et al. 2001, Gillanders 2005, Vasconcelos et al. 2008, Chittaro et al. 2009). *P. flesus* and *D. labrax* have spatially segregated adult and juvenile life stages along the Portuguese coast: adults live in the marine environment and spawn offshore, larvae are transported towards the coast and post-larvae or early juveniles enter estuaries and concentrate in nursery grounds in late spring, where they remain their first year of life, and up to two years, dispersing afterwards to deeper areas and migrating out to coastal waters (Cabral & Costa 2001, Cabral et al. 2007, Martinho et al. 2008, Dolbeth et al. 2008, Vasconcelos et al. 2010). The distinct elemental fingerprints among estuaries can be used to address the movement of these fish to coastal waters and ultimately understand the effective nursery role of estuaries as postulated by Beck et al. (2001).

Most elements showed significant differences among estuaries and sampling times, which are likely a reflection of the observed variability in environmental conditions. Hence, otolith elemental fingerprints are not likely to remain consistent through time, both among years as well as at smaller temporal scales, such as months or seasons (Gillanders 2002, Hamer et al. 2003, Clarke et al. 2009, Walther & Thorrold 2009). If elemental tags are assumed to be constant, when in fact they shift over time, temporal variations may confound spatial differences, resulting in incorrect estimates of the relative contribution of estuarine nurseries (Gillanders 2002) with some areas potentially being overlooked or their prominence at least downplayed. The overlap in the elemental fingerprints of different years between the Mondego and Ria de Aveiro, for *P. flesus*, and Tejo and Ria de Aveiro, for *D. labrax* observed in this study suggest this is possible. Knowledge of inter-annual variation of these natural tags is key to establish baseline data to retrospectively determine estuarine origin of adult fish from coastal populations. Since estuarine otolith elemental tags were found to be year specific, elemental fingerprints used to retrospectively classify coastal adults must be from juveniles of the same year classes of adult fish (Gillanders 2005, Chittaro et al. 2009).

Results suggest that estuarine juvenile elemental fingerprints be characterised on a yearly basis, compiling a library of reference elemental tags to be later used to determine the nursery origin of coastal adult fish. This is reinforced by the high misclassification rates obtained when pooling annual data or using years as proxies to classify other years, as even in cases when inter-annual variations in elemental tags seemed lower (e.g. in the canonical variate plots), elemental fingerprints were still significantly different and year specific. Current inter-annual comparisons were made with a three year gap. Nonetheless, elemental fingerprints of *Menidia menidia* collected in coastal marine areas in adjacent years also varied significantly (Clarke et al. 2009), whilst elemental fingerprints of whole otoliths of marine *Gadus morhua* were similar in contiguous years, but differed markedly over broader time intervals (Campana et al. 2000). A similar pattern was observed in otolith elemental fingerprints of *Pagrus auratus* from coastal

bays between adjacent year classes and over longer time periods (Hamer et al. 2003).

Seasonal variation in otolith elemental fingerprints was found for *P. flesus* and *D. labrax*. For both species, individuals remain in the same estuaries for several months where they may experience considerable environmental variation that can lead to differences in otolith elemental chemistry. Even at this shorter time scale (months or seasons) the observed temporal variation can have a confounding effect on the accurate discrimination of estuaries. Yet, similar to Thorrold et al. (1998) and Hamer et al. (2003) who analysed two months within a three month period, variations in the elemental fingerprints among seasons were of minor influence to the overall spatial discrimination of estuaries when data from the different sampling seasons were pooled. Ultimately, attaining elemental fingerprints that characterize the estuarine nursery of age-0 juveniles, via integration of seasonal variation, is a great advantage in the context of connectivity assessments. To determine juvenile nurseries of adult fish will require matching portions of otolith corresponding to that specific time period and it is unlikely that the juvenile section of adult otoliths can be specifically matched to a particular month within the year. Moreover, such precise temporal matching of adults to juvenile signatures would be particularly problematic for other species that have protracted recruitment seasons with multiple cohorts, where mismatches between early and late recruits could occur.

The temporal scale of interest to assess connectivity in these marine migrant species should be year class. Therefore, it seems best to define baseline natural tags that are representative of an entire year class of juveniles integrating seasonal variation. Present results advise that such signatures can be established via multiple sampling throughout the main period of occurrence of age-0 juveniles within juvenile areas. Or, if only one sampling period is carried out, it is probably best that it takes place later in the year and more otolith material is analysed by using larger spot sizes encompassing the estuarine life period of these juvenile fish. Alternatively, multiple readings, or profiles, along segments of the otolith growth axis could account for seasonal variation. In general, additional data enhances classification success as long as, in this case, the elemental signatures lack covariance over time (Fabrizio 2005, White & Ruttenberg 2007). Integrating seasonal variation to define fingerprints that characterize an entire year class also consolidates overall classification accuracy by reducing the likelihood that spatial discrimination is based on a single period which may haphazardly have a larger overlap in elemental fingerprints and consequently lesser discriminatory power. Recognizing that juvenile fish use estuarine habitats for a prolonged period, successful incorporation of temporal variation in elemental fingerprints can alter the scale at which connectivity is assessed, lead to more comprehensive estuarine natural tags, and accurate descriptions of connectivity and recruitment estimates.

Overall, different years or seasons produced higher classification accuracies than others. The chemical composition of otoliths is influenced by several factors, including environmental conditions (water chemistry, salinity, temperature) (Bath et al. 2000, Martin & Thorrold 2005, Miller 2011) as well as physiology and growth (Kalish 1991, Hamer & Jenkins 2007). Most elements showed significant differences among estuaries and sampling times that are likely due

to differences in water chemistry, salinity and temperature (reviewed in Elsdon et al. 2008) as these vary greatly in dynamic systems such as estuaries. Yet, despite variations throughout 2009, no conspicuous patterns emerged between temperature or salinity and otolith composition. Notwithstanding, the Mira estuary attained highest Mn:Ca which are probably related to an iron-manganese ore mining facility near the water basin. Hence, as most estuarine nurseries are affected by anthropogenic impacts, knowledge of water and sediment contamination may highlight potential elements for discrimination purposes. Additionally, temperature is likely to influence growth rates and regulate other physiological aspects (e.g. otolith precipitation rates). In fact, growth, otolith precipitation and temperature are highly correlated, so it is difficult to address their effects independently in the field (Martin & Thorrold, 2005). Though differences in growth rates among estuaries cannot be discounted as a potential source for otolith composition variation, ontogenetic effects are minimized by analysing fish within the same life period and with similar estuarine residence times (Gillanders 2002). Elemental fingerprints have also been shown to be species specific, as a result of differences in phylogeny, physiology and ecology (Gillanders & Kingsford 2003, Swearer et al. 2003, Reis-Santos et al. 2008), and varied between flounder and sea bass in the estuaries where they co-occurred. Differences, particularly between Pleuronectiforme and Perciforme fish, are likely due to distinct physiological regulation, habitat use patterns and diet (see Reis-Santos et al. 2008). Furthermore, age-0 juveniles often have segregated nursery grounds within an estuary (e.g. Mondego) and so are subject to distinct environmental patterns (Martinho et al. 2007, Vasconcelos et al. 2010). Overall, it is the complex relationship between water chemistry, temperature and salinity on otolith chemistry, influenced by species' ecological and physiological responses that contribute to the distinct elemental fingerprints, spatially and temporally. Evaluating how different factors affect elemental incorporation can be addressed via experimental work (e.g. Martin & Thorrold 2005, Miller 2011) also aiming at identifying elements that may be more robust at discriminating among estuaries or environments. Overall, knowledge on the factors that influence otolith chemistry is far from complete, and identified environmental effects are often difficult to generalize, as they differ among species and environments. However, understanding the mechanisms underlying elemental incorporation is not required to use elemental differences in otoliths as natural tags (Thorrold et al. 1998, Elsdon et al. 2008). The approach rests on differences in otolith chemistry among individuals from different estuaries that can then be treated as discrete groups, regardless of the mechanisms generating variation.

The application of elemental fingerprints as a baseline for connectivity must move beyond just establishing spatial differences among estuaries but also understand the natural variability in chemical signatures over time, thus defining elemental tags that can effectively be used in the long-term. A key aspect is to promote processes that encompass both spatial and temporal changes in the natural tags whilst taking into account the natural history of the organisms being analysed (Cook 2011), namely by characterizing otolith elemental fingerprints on a yearly basis, and taking into account the main period juveniles remain in estuarine nurseries. Ultimately, it is

essential to strive towards understanding the factors that influence the application of otolith chemistry to address connectivity as basis for the development of spatial management measures. Results of the current study highlight temporal differences in the otolith chemistry of age-0 juveniles of *P. flesus* and *D. labrax* in estuarine nurseries; as well as the need to adequately incorporate temporal variation in elemental fingerprints to establish year specific libraries of baseline natural tags. This will be vital for the retrospective determination of the estuarine nursery of coastal adult fish. Estimating the relative contributions of estuarine nurseries to adult populations of marine migrant fish has the potential to aid the development of integrated fisheries and coastal management plans, as well as prioritizing conservation efforts. Understanding the spatio-temporal patterns of fish population connectivity is paramount towards establishing spatially explicit management plans as measures should ideally be dictated by the dispersal and connectivity of multiple species.

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## CHAPTER 5

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### **Connectivity within estuaries: an otolith chemistry and muscle stable isotope approach**

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In review in Marine Biology

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## Connectivity within estuaries: an otolith chemistry and muscle stable isotope approach

**Abstract:** Understanding movements and habitat use patterns of marine migrant and estuarine resident fish within estuaries has major implications for habitat conservation and management. Muscle stable isotopes ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) and otolith elemental composition (Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca and Pb:Ca) were used to evaluate connectivity between two separate estuarine areas in summer and autumn for juvenile age-0 marine migrant *Dicentrarchus labrax* and estuarine resident *Pomatoschistus microps*. Distinct isotopic and otolith elemental signatures were found between estuarine areas for *D. labrax* and *P. microps*, and in both sampling times. High classification accuracies to collection sites were achieved via otolith elemental signatures (80 % to 94 %), and a combined analysis using both muscle stable isotopes otolith chemistry resulted in increased accuracy with no classification errors. Overall, low site connectivity was found for both species. The use of two distinct natural tags provided corroborative and complementary information on fish movement and intra-estuarine habitat use at different temporal scales, whilst elucidating distinct ecological and environmental linkages. Ultimately, the combined use of distinct natural tags showed great promise to unravel intra-estuarine connectivity patterns.

**Keywords:** Natural tags; movement; nursery; habitat use; fish; Portugal

### 1. Introduction

Estuaries have long been regarded as highly productive and valuable ecosystems. They are essential to many marine migrant fish species which use particular habitats or sites as nursery areas, where juveniles remain for protracted periods ranging from months to years before recruiting to coastal adult populations (Beck et al. 2001, Able 2005). Likewise, resident fish species, those which complete their entire life cycle within an estuary, rely on a range of habitats and play a fundamental role in the overall dynamics and functioning of estuarine systems (Elliott & Dewailly 1995, Dolbeth et al. 2007, França et al. 2009). Yet, detailed knowledge on the extent to which individual fish occupy specific sites is lacking. Moreover, the movement and temporal habitat use patterns of estuarine and juvenile marine migrant fish among segregated habitats within estuaries is largely unknown (Able et al. 2005, Able et al. 2012, Green et al. 2012).

A better understanding of whether fish move among segregated estuarine areas and habitats or show high site fidelity has major implications for habitat conservation, resource management and the safeguard of estuarine ecological integrity. Knowledge of the connectivity among habitats is in keeping with the need to identify ecologically important habitats, and is particularly critical considering that estuaries are threatened by extensive habitat loss and degradation due to widespread anthropogenic pressures (Kennish 2002, Vasconcelos et al. 2007, Courrat et al. 2009).

Unravelling fish movement is challenging but has been tackled by a variety of approaches from abundance and size-frequencies distributions to artificial and natural tagging methods (Gillanders 2009). Conventional tagging methods are logistically difficult to use on juveniles and

early life history stages due to their reduced size, high mortality and low recapture rates. Hence, natural tags are increasing in their use. Among these, tissue stable isotope ratios of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) (Herzka 2005, Trueman et al. 2012) and otolith chemical composition (Elsdon et al. 2008, Sturrock et al. 2012) are common tools in assessing fish movement and habitat use.

Stable isotope analysis has been used to examine movement to, from and within estuaries (Hobson 1999, Herzka 2005, Trueman et al. 2012). The premise for its use is based on primary producer groups exhibiting distinctive isotopic signatures that are propagated through local food webs; and if food sources are distinct regarding their isotopic signatures, fish feeding in these habitats will also have distinct isotopic signatures. Overall, the stable isotope signature of an organism's tissue reflects its assimilated diet, spatial variation of food webs and local biogeochemistry, and can be used to infer geographic origin, habitat use and site fidelity (e.g. Vinagre et al. 2008, Green et al. 2012) as well as unravel connectivity and trace movement patterns (e.g. Suzuki et al. 2008, Verweij et al. 2008). Non-migratory individuals are expected to exhibit similar stable isotopic signatures, in equilibrium with local food webs, while transient individuals moving between habitats should display intermediate or greater isotopic variation (Fry et al. 2003, Rubenstein & Hobson 2004, Herzka 2005).

The chemical composition of otoliths has been widely used to assess population structure (e.g. Thresher & Proctor 2007, Tanner et al. 2012), reconstruct migration patterns (e.g. Hamer et al. 2006, Morales-Nin et al. 2012), identify estuarine nurseries (e.g. Gillanders & Kingsford 2000, Reis-Santos et al. 2012), and assess connectivity between juvenile and adult populations (e.g. Vasconcelos et al. 2008, Chittaro et al. 2009). The use of otoliths as a natural tag is possible due to their continuous growth, metabolic inertness and the fact that trace element incorporation is influenced by physical and chemical properties of the surrounding water (Campana 1999). Hence, over time, fish that occupy different estuarine sites or habitats may be expected to have distinct otolith elemental compositions. However, successful application of otolith elemental signatures depends on their variation at relevant spatial scales; and several studies have shown that sites within estuaries can be discriminated (Gillanders & Kingsford 2000, Miller 2007, Chittaro et al. 2009, Tanner et al. 2011).

Despite the increased application of natural markers, studies using tissue stable isotopes and otolith chemistry to assess connectivity or population structure are scarce (but see Verweij et al. 2008, Lawton et al. 2010, Dierking et al. 2012) with no studies combining tissue isotope and otolith chemistry in an integrated manner. Movement and habitat use patterns of fish within estuaries may vary as a function of life history strategies, size- or age- specific habitat preferences and also be influenced by a combination of behavioural, ecological and environmental factors (e.g. salinity, temperature, food availability) (Stoner et al. 2001, Vasconcelos et al. 2010). Individuals respond to these processes at distinct spatio-temporal scales and therefore the study of movement and connectivity in estuarine fish may benefit from combined approaches of muscle stable isotope ratios and otolith chemistry. The use of multiple distinct natural markers is expected to enhance connectivity assessments as they potentially

respond at different spatio-temporal scales (e.g. Thorrold et al. 2002, Abaunza et al. 2008, Verweij et al. 2008, Lawton et al. 2010, Perrier et al. 2011). For example, tissue isotopes are expected to integrate information over days to months (Rubenstein & Hobson 2004, Herzka 2005), whereas otoliths incorporate temporally resolved information throughout fish's life history (Campana 1999, Elsdon et al. 2008).

The aim of the present study was to use muscle stable isotopes and otolith chemistry to evaluate the connectivity between segregated estuarine areas for juvenile age-0 marine migrant sea bass *Dicentrarchus labrax* and estuarine resident common goby *Pomatoschistus microps*. Specifically, we examined if tissue stable isotopes ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) and otolith elemental composition varied between estuarine sites over time and if individuals from each site could be distinguished. We also examined whether the combined use of these techniques enhanced our ability to unravel intra-estuarine connectivity, and evaluated the complementarity between these two natural tags.

## 2. Material and methods

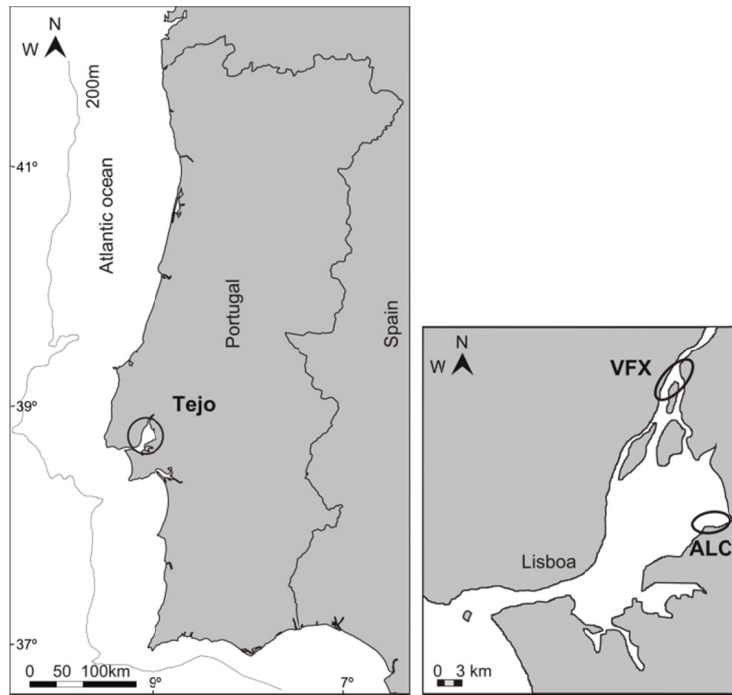
### 2.1. Study area

Samples were collected from the Tejo estuary, a large partially mixed estuary of 320 km<sup>2</sup>, and tidal amplitude of c. 3 m. In the present study, two distinct sites were sampled: Vila Franca de Xira (VFX) in the upper estuary and Alcochete (ALC) in the middle reaches of the estuary (Figure 1). These sites are the two acknowledged nursery areas for juveniles of several marine migrant fish species in the Tejo estuary, including *D. labrax* (e.g. Cabral & Costa 2001, Vasconcelos et al. 2010). Both sites are fringed by extensive salt marshes, and are the main feeding and shelter habitats for the estuarine resident *P. microps* (e.g. Salgado et al. 2004). The latter is a key benthic predator and an important species within estuarine salt marsh systems (Cattrijse & Hampel 2006), as well as the most abundant fish in the Tejo estuary (França et al. 2009).

### 2.2. Sample collection and preparation

Beam trawls were conducted at both sites in July (early summer) and October (early autumn) 2009 to capture age-0 *D. labrax* and *P. microps*. Once collected, fish were transported on ice and frozen at the laboratory (-20 °C). All fish were measured (total length to the nearest mm) (Table 1). For stable isotope analysis dorsal white muscle samples were extracted as  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  tend to be less variable in this tissue (Pinnegar & Polunin 1999). Analyses were carried out for each individual fish for *D. labrax*. For *P. microps*, between eight and ten individual fish were pooled into a single sample due to their small size. For both species five replicates were run per site and sampling time. All instruments and tissue used in sample preparation for stable isotope analyses were cleaned with deionised water. For otolith chemistry, sagittal otoliths were extracted (c. 15 fish per site and season for each species, including those used for stable isotope analysis), washed and cleaned of adhering tissue with ultra-pure water and allowed to air dry in a positive pressure laminar flow hood.





**Figure 1.** Location of the Tejo estuary and sampled estuarine nursery grounds sites of Vila Franca de Xira (VFX) and Alcochete (ALC).

**Table 1.** Mean total length (and standard error) in mm of *Dicentrarchus labrax* and *Pomatoschistus microps* collected in July (early summer) and October (early autumn) at each estuarine site (ALC - Alcochete; VFX - Vila Franca de Xira) and used for stable isotope and otolith elemental composition analysis.

Species	Site	Average length	
		July	October
<i>D. labrax</i>	ALC	67 (1)	108 (2)
	VFX	62 (2)	111 (3)
<i>P. microps</i>	ALC	40 (3)	37 (2)
	VFX	32 (1)	38 (1)

### 2.2.1. Stable isotope analysis

Standard preparation for stable isotope analysis consisted of drying samples in an oven at 60 °C until constant weight. Dried tissues were then ground to a fine powder with a mortar and pestle.

Prior to carbon stable isotope analysis, it is recommended that carbonates are removed from samples, as these have different origins from the carbon found in tissues and may bias isotope analysis (Ng et al. 2007). Hence, an acidification trial was run to ascertain potential carbonate contamination on a subsample of the analysed tissues. Subsamples were acidified with drops of 10 % HCl while being observed under a dissecting microscope. If bubbling occurred, the whole subsample was acidified, rinsed with distilled water, re-dried at 60 °C, and stored in glass vials. Subsamples of *P. microps* were acidified for subsequent  $^{13}\text{C}$  quantification.

$^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$  ratios were determined by continuous flow isotope ratio mass spectrometry (CF-IRMS), on an Isoprime (Gv, Uk) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. The standards used were IAEACH6 and IAEA-CH7 or IAEA-600 for carbon isotope ratio, and IAEA-N1 and IAEA-600 for nitrogen isotope ratio. Isotope ratios were measured relative to the international standards of PeeDee Belemnite for  $\delta^{13}\text{C}$  and atmospheric  $\text{N}_2$  for  $\delta^{15}\text{N}$ . Precision of the mass spectrometer, calculated using values from duplicate samples was  $<0.2\text{‰}$ . Stable isotope ratios were expressed using the delta ( $\delta$ ) notation representing parts per thousand differences from a standard reference material:

$$\delta X = [ ( R_{\text{sample}} / R_{\text{standard}} ) - 1 ] \times 10^3$$

where X is  $^{13}\text{C}$  or  $^{15}\text{N}$ ; and R is the ratio of heavy to light isotopes ( $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ ).

### 2.3. Otolith chemistry analysis

Otolith sample preparation and analytical procedures followed Reis-Santos et al. (2012). Briefly, the right otolith of each fish was embedded in EpoFix resin (Struers), spiked with indium ( $^{115}\text{In}$ ) at 30 ppm to allow discrimination between otolith material and resin during analysis. Otoliths were sectioned transversely through the nucleus using a low-speed saw (Buehler Isomet). Sections were polished to c. 250  $\mu\text{m}$  with lapping film (30  $\mu\text{m}$ , 9  $\mu\text{m}$  and 3  $\mu\text{m}$  grit size successively) and mounted on glass slides with indium-spiked thermoplastic glue (CrystalBond 509). Slides were then cleaned, sonicated and triple-rinsed with ultrapure water, dried under a laminar flow hood and stored individually in sealed plastic bags.

A New Wave 213 nm UV high performance (Nd:YAG) laser microprobe coupled to an Agilent 7500cs inductively coupled plasma mass spectrometer (ICP-MS) was used to quantify  $^7\text{Li}$ ,  $^{24}\text{Mg}$ ,  $^{43}\text{Ca}$ ,  $^{55}\text{Mn}$ ,  $^{65}\text{Cu}$ ,  $^{88}\text{Sr}$ ,  $^{138}\text{Ba}$  and  $^{208}\text{Pb}$  elemental concentrations in otoliths. Ca was used as an internal standard to correct for variation in ablation yield between samples.  $^{115}\text{In}$  was also measured but solely as a marker to discriminate between spiked resin, or CrystalBond, and otolith matrices.

Otoliths were analysed using a 30  $\mu\text{m}$  spot on the outside edge of the otoliths to quantify recent elemental incorporation. Otoliths were analysed in a random order and elemental data were collected from the same region for each otolith. A certified glass reference standard NIST 612 (National Institute of Standards and Technology) was run at the start and end of each session and after every 10 otoliths to correct for mass bias and machine drift. External precision (% relative standard deviation) was assessed by measurements of a calcium carbonate certified reference material MACS-3 (United States Geological Survey) and were as follows: 4.1 % (Li), 5.1 % (Mg), 4.2 % (Mn), 7.4 % (Cu), 2.4 % (Sr), 3.2 % (Ba), and 7.1 % (Pb). Data reduction, including background corrections, mass count data conversion to concentrations (ppm) and limits of detection were all performed for each individual sample via Glitter software (GEMOC, Macquarie University, Sydney, Australia). Resulting otolith elemental concentration data were converted to molar concentrations and standardised to calcium (element:Ca). All further otolith chemistry data analyses were carried out on  $\log(x+1)$  transformed element:Ca data.

## 2.4. Data analysis

Differences in fish length, muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotopic signatures as well as otolith elemental signatures were evaluated via permutational analysis of variance (PERMANOVA) using Euclidean distance dissimilarity matrices. Species were analysed separately. If significant differences occurred, *a posteriori* pairwise tests were used to assess which sites or times differed. The quantitative nature of stable isotope analysis and otolith chemistry enabled the use of both datasets singly as well as in combination.

For the analysis of each natural marker individually,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  plots were used to evaluate patterns of variation in the isotopic signatures of *D. labrax* and *P. microps* per site and time, whilst canonical variate plots of 95 % confidence limits around the mean were used to display the variation in the otolith elemental signatures between sites according to sampling time in reduced space. Canonical analysis of principal coordinates (CAP) was used to assess the accuracy of otolith elemental signatures in classifying fish to their collection site. CAP is a constrained ordination for discriminating among *a priori* groups, and the leave-one-out approach was used to provide a sound and unbiased measure of how distinct groups are in multivariate space (Anderson & Willis 2003). Cross-validated classification accuracies per site and sampling time were determined for age-0 *D. labrax* and *P. microps* as an indicator of whether juveniles remained within each of the intra-estuarine sites.

Upon their analysis singly, we explored the combined value of these techniques to unravel intra-estuarine connectivity using the individuals for which we had corresponding stable isotope analysis and otolith chemistry data. In the case of *P. microps*, where muscle samples had to be pooled to allow stable isotope analysis, individual otolith elemental chemistry data was randomly pooled into five replicates, in order to analyse them in the same way as the isotope data. Muscle stable isotopes and otolith elemental composition data were used in a CAP analysis to compare their single and combined value to assign individual samples to estuarine sites. Cross-validated classification accuracies per site and sampling time were determined. In addition, stable isotope scores and otolith elemental scores were determined from canonical coordinates in multivariate space and then combined graphically, following Perrier et al. (2011). First, for each natural tag, the centroid coordinates of each site were determined as the mean value of the axes in canonical variate plots (X, Y, Z – or up to 100 % of the variability explained). Then the distances (*D*) of the individual sample (*i*) to the centroids of both sites (ALC and VFX) were calculated:

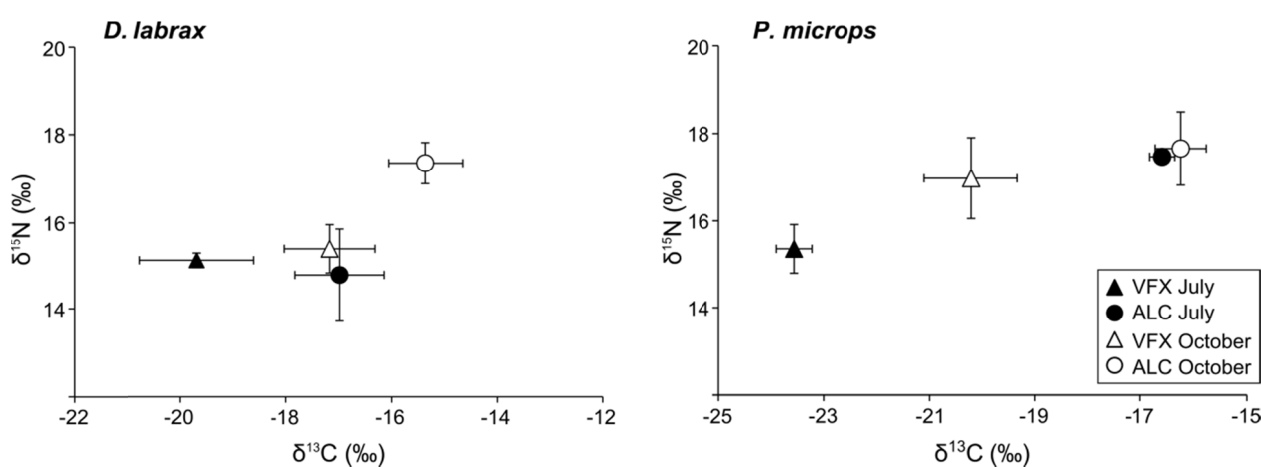
$$D_{i,Site} = \sqrt{(X_i - X_{Site})^2 + (Y_i - Y_{Site})^2 + (Z_i - Z_{Site})^2}$$

where  $X_i$ ,  $Y_i$  and  $Z_i$  are individual (*i*) coordinates, and  $X_{site}$ ,  $Y_{site}$ ,  $Z_{site}$  the coordinates of the centroid of each site. In the end a score for each individual ( $S_i$ ) was calculated, in relation to site ALC, as:  $S_i = D_{ALC} / (D_{ALC} + D_{VFX})$ . Consequently scores closer to zero were indicative of individuals traced back to ALC whilst scores closer to 1 indicated individuals traced back to VFX. Scores were calculated for each natural tag and combined in scatterplots.

### 3. Results

All *D. labrax* were age-0 juveniles, increasing in length between the two sampling times in both nursery sites (Table 1). Among sites, sea bass were of similar lengths, even though there was a significant difference between the two sites in July ( $t = 3.11$ ,  $p < 0.05$ ). *P. microps* were similar in size between sites and sampling times, with the exception of gobies from one site (VFX) in July which were significantly smaller ( $t = 4.21$ ,  $p < 0.001$ ).

Isotopic signatures of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  varied between sites and sampling times for both species (*D. labrax*: Site x Time interaction, Pseudo-F = 4.43,  $p < 0.05$ ; *P. microps*: Site x Time interaction, Pseudo-F = 15.02,  $p < 0.001$ ) but their magnitude of variation differed (Figure 2). Pairwise tests confirmed differences between sites within each sampling season (*D. labrax*: in July,  $t = 2.29$ ,  $p < 0.05$ , and in October,  $t = 4.68$ ,  $p < 0.001$ ; *P. microps*: in July,  $t = 22.52$ ,  $p < 0.01$ , and in October,  $t = 4.98$ ,  $p < 0.01$ ). Variations in isotopic signatures per site and time were smaller than those between sites for each species. In general, individuals collected in ALC were more enriched in  $^{13}\text{C}$  and  $^{15}\text{N}$  for each sampling time, with the exception of  $\delta^{13}\text{C}$  in *D. labrax* from July. Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ranged from -15.35 to -19.69 ‰ ( $\delta^{13}\text{C}$ ) and 14.78 to 17.40 ‰ ( $\delta^{15}\text{N}$ ) in *D. labrax*; and from -16.24 to -23.56 ‰ ( $\delta^{13}\text{C}$ ) and 15.35 to 17.66 ‰ ( $\delta^{15}\text{N}$ ) in *P. microps*. No overlap in fish isotopic signatures between sites per sampling time was observed for either of the species.



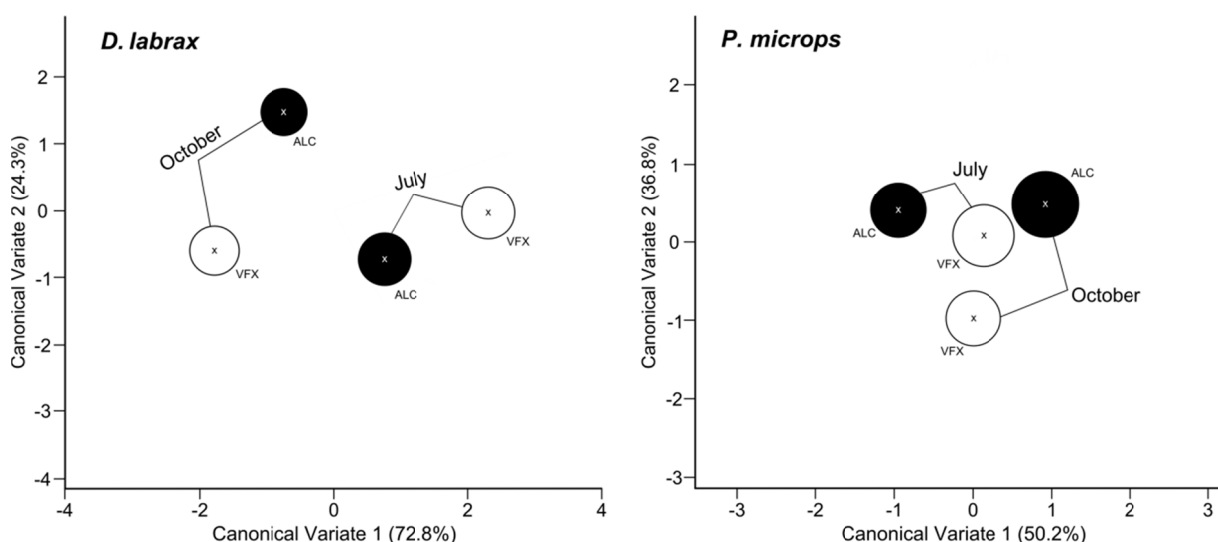
**Figure 2.** Plots of mean (and standard deviation) muscle stable isotope composition ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of *Dicentrarchus labrax* and *Pomatoschistus microps* collected in estuarine sites VFX (triangles) and ALC (circles) in July (full symbols) and October (open symbols).

Element:Ca concentrations in otoliths of *D. labrax* and *P. microps* varied among estuarine sites and sampling times (Table 2, Figure 3). Comparison of the multi-element estuarine otolith signatures showed a significant interaction between site and sampling time for each species (*D. labrax*: Site x Time interaction, Pseudo-F = 2.60,  $p < 0.05$ ; *P. microps*: Site x Time interaction, Pseudo-F = 7.70,  $p < 0.001$ ). Pairwise *post hoc* tests showed differences between the two sites within each sampling time (*D. labrax*: in July,  $t = 1.77$ ,  $p < 0.05$ , and in October,  $t = 2.25$ ,  $p < 0.001$ ; *P. microps*: in July,  $t = 1.81$ ,  $p < 0.01$ , and in October  $t = 2.75$ ,  $p < 0.001$ ). For individual

element:Ca ratios, univariate PERMANOVA showed significant differences between sites in at least one of the sampling times for Li:Ca, Mg:Ca, Ba:Ca and Pb:Ca in both species, as well as for Sr:Ca in *D. labrax* and Mn:Ca in *P. microps*. Overall, differences in fish otolith chemistry between sites were evident in the canonical variate plots, with no overlap present. Nonetheless, canonical separation between sites was larger in October in comparison to July for both species; and multi-element signatures of *P. microps* were in general clustered closer together in the canonical space (Figure 3).

**Table 2.** Mean (and standard error) element:Ca ratios of Li, Mg, Mn, Cu, Sr, Ba and Pb in otoliths of *Dicentrarchus labrax* and *Pomatoschistus microps* collected in July and October in each estuarine site (ALC – Alcochete; VFX – Vila Franca de Xira). [All element:Ca data are in  $\mu\text{mol} \cdot \text{mol}^{-1}$ , except Sr:Ca ( $\text{mmol} \cdot \text{mol}^{-1}$ ) and Pb ( $\text{pmol} \cdot \text{mol}^{-1}$ )]

Species	Time	Site	Otolith Chemistry						
			Li:Ca	Mg:Ca	Mn:Ca	Cu:Ca	Sr:Ca	Ba:Ca	Pb:Ca
<i>D. labrax</i>	July	ALC	3.34 (0.26)	188.94 (7.99)	5.59 (0.47)	0.59 (0.13)	1.90 (0.04)	2.36 (0.17)	20.78 (2.23)
		VFX	2.73 (0.24)	179.71 (12.42)	7.47 (0.89)	0.51 (0.08)	1.86 (0.04)	3.56 (0.29)	13.28 (2.15)
	October	ALC	2.18 (0.14)	103.21 (3.48)	2.84 (0.48)	0.52 (0.09)	1.96 (0.04)	2.10 (0.12)	30.13 (2.37)
		VFX	2.56 (0.31)	125.56 (7.81)	3.24 (0.42)	0.44 (0.08)	1.85 (0.03)	1.30 (0.07)	21.56 (3.24)
<i>P. microps</i>	July	ALC	3.21 (0.10)	81.51 (4.02)	2.80 (0.40)	0.53 (0.22)	2.52 (0.05)	1.21 (0.09)	15.49 (1.39)
		VFX	2.78 (0.19)	64.88 (5.03)	2.41 (0.38)	0.48 (0.10)	2.41 (0.06)	1.36 (0.16)	9.61 (1.23)
	October	ALC	2.26 (0.39)	83.30 (6.82)	3.62 (0.92)	0.56 (0.09)	2.34 (0.16)	1.32 (0.06)	5.53 (0.92)
		VFX	2.70 (0.23)	69.64 (3.11)	1.47 (0.21)	0.44 (0.07)	2.41 (0.05)	1.10 (0.11)	10.61 (0.85)



**Figure 3.** Canonical variate plots summarising variation in otolith multi-elemental signatures of *Dicentrarchus labrax* and *Pomatoschistus microps* between estuarine sites VFX (open) and ALC (full) in July and October. Ellipses represent 95 % confidence limits for the mean.

Results of CAP analysis on otolith chemistry revealed that *D. labrax* and *P. microps* could be classified to their estuarine collection site with an elevated degree of accuracy (Table 3). For *D. labrax*, classification accuracies to their estuarine nursery areas ranged between 80.0 % and 94.2 %. Overall cross validated *D. labrax* were correctly classified 83.3 % and 87.9 % in July and October, respectively. Similarly high classification accuracies were also found for *P. microps*, with 85.3 % and 89.7 % overall cross validated correctly classified individuals in July and October, respectively.

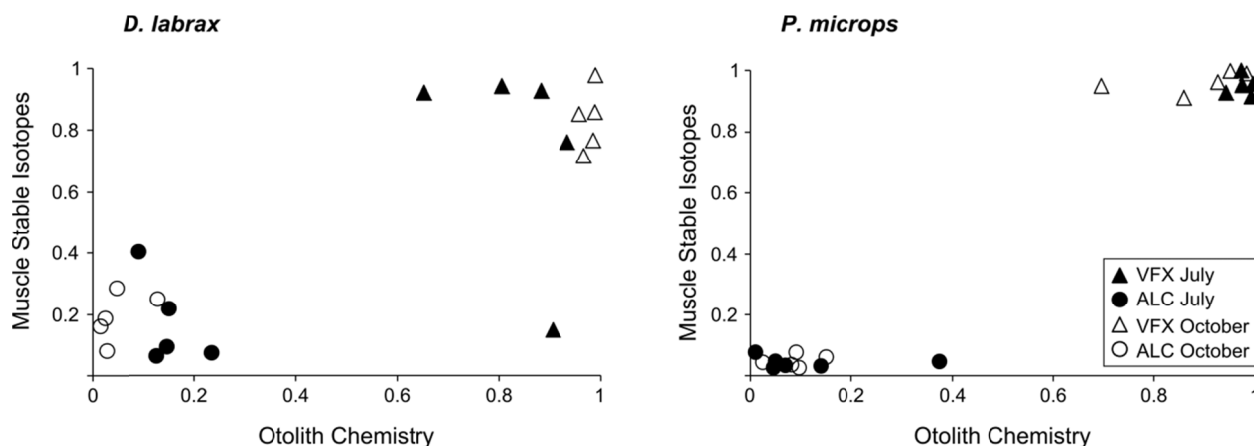
**Table 3.** Summary of correct cross-validated classified individuals of *Dicentrarchus labrax* and *Pomatoschistus microps* to their estuarine collection site (ALC – Alcochete; VFX – Vila Franca de Xira) in July and October. Classification was based on otolith multi-elemental signatures and canonical analysis of principal components. Sample size is also shown (n).

Species	Time	Cross validated correctly classified individuals (%)			(n)
		ALC	VFX	Overall	
<i>D. labrax</i>	July	86.7	80.0	83.3	30
	October	94.2	81.3	87.9	33
<i>P. microps</i>	July	88.3	82.4	85.3	34
	October	85.7	93.3	89.7	29

Combining the two natural tags (muscle stable isotopes and otolith chemistry), even if for a restricted number of specimens, improved or maintained overall classification accuracies (Table 4). CAP analysis integrating both data sets resulted in 100 % classification accuracies for both species. The integrated plots of individual scores confirmed that fish from each site displayed generically distinct muscle isotopic and otolith elemental signatures (Figure 4). Results follow the patterns found within the single assessments for each technique, with larger variations in scores in *D. labrax*, compared to *P. microps*, and in July, compared to October. The plots highlight one individual of *D. labrax*, collected in VFX in July, which presented a muscle isotopic score that closely resembled that of ALC, whilst its otolith chemistry matched those for its collection site.

**Table 4.** Summary of total correct cross-validated classifications of *Dicentrarchus labrax* and *Pomatoschistus microps* to estuarine collection sites (ALC and VFX) in July and October, based on individual's otolith elemental signatures, tissue stable isotopic signatures and a combined approach (n = 5 per site and time). Classifications were achieved via canonical analysis of principal components.

Species	Time	Total cross validated correctly classified individuals (%)		
		Otolith Chemistry	Tissue Stable Isotopes	Combined
<i>D. labrax</i>	July	80	90	100
	October	90	100	100
	Overall	85	95	100
<i>P. microps</i>	July	80	100	100
	October	100	100	100
	Overall	90	100	100



**Figure 4.** Plot combining otolith elemental scores and stable isotope scores for individuals of *Dicentrarchus labrax* and *Pomatoschistus microps* collected in VFX (triangles) and ALC (circles) in July (full symbols) and October (open symbols). Scores close to 0 were indicative of individuals traced back to ALC whilst scores closer to 1 are indicative of individuals with isotopic or elemental signature scores traced back to VFX.

#### 4. Discussion

Distinct tissue isotopic and otolith elemental signatures were found between the two sampling sites and times for *D. labrax* and *P. microps*. These results were indicative that over the study period (i.e. in early summer and early autumn) juvenile age-0 marine migrant *D. labrax* and estuarine resident *P. microps* had low connectivity between the two sampled estuarine sites within the Tejo estuary. The combined use of two natural tags provided further corroborative and complementary evidence on the low connectivity between estuarine sites.

Lower  $\delta^{13}\text{C}$  values were found in the upper estuary (VFX), which is in agreement with expected natural patterns of  $\delta^{13}\text{C}$  variation that display an enrichment trend along the terrestrial-estuarine-marine gradient (Fry 2002). Terrestrial primary organic sources and C3 halophytes have more depleted  $\delta^{13}\text{C}$  values than marine producers (Fry & Sherr 1984, Herzka 2005). In contrast, considerably richer  $\delta^{15}\text{N}$  values than those found in fish along the coastal environment (c. 7 to 10 ‰) (Vinagre et al. 2011), were likely due to the anthropogenic nitrogen inputs in the estuary (e.g. wastewater, fertilizers) (Herzka 2005, Schlacher et al. 2005, Dierking et al. 2012).

One of the challenges in stable isotope approaches is to establish if the species of interest are using or moving between food webs that differ in isotopic composition (Hobson 1999). The observed intra-estuarine differences in fish muscle stable isotopes were a reflection of the isotopic composition of local food webs and available prey; previously characterized in França et al. (2011). Two isotopically distinct food webs were identified for these estuarine nursery sites, based on  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of a variety of producers, primary consumers and fishes. Variations in food web dynamics and estimates of the relative importance of organic matter sources, determined using mixing models, outlined the contribution of local benthic pathways to spatial variation in isotopic composition. Moreover, these same estuarine sites were regarded as separate feeding locations and the importance of local macroinvertebrate prey for *D. labrax* and *P. microps* was established via the mixing models (França et al. 2011). The latter support

our findings regarding distinct isotopic signatures and the low connectivity estimates between sites.

The smaller size of individuals of *P. microps* collected in VFX in July is the likely cause for the lower  $\delta^{15}\text{N}$  values measured. *P. microps* undergo a diet shift from meiofauna to macrofauna prey at c. 30 mm of length (Jackson & Rundle 2008), hence smaller individuals occupy lower trophic positions and consequently have lower  $\delta^{15}\text{N}$ , as  $\delta^{15}\text{N}$  ratios increase with consumer trophic levels (Post 2002, Pasquaud et al. 2010).

Despite the distinct life history strategies, a low level of connectivity and elevated site fidelity between sites was found for both *D. labrax* and *P. microps*. This was similar to the findings of Green et al. (2012), among salt marshes in estuarine environments for multiple fish species, including sea bass and common goby. Nonetheless, Vinagre et al. (2008, 2010), highlighted an overlap in fish isotopic signatures for age-1 Senegalese sole *Solea senegalensis* between the same two sites in the present study. These transient and intermediate isotopic signatures, in individuals that remained within the estuary over winter, supported increased connectivity among intra-estuarine sites for larger fish. Thus, suggesting that ontogenetic changes in movement patterns and site fidelity occur as fish mature, most likely due to increased movement capacity and foraging areas, larger energy demands, and potential changes in environmental tolerances (Primo et al. 2013). However, in the current study, there was no evidence of increased connectivity or a decrease in isotopic distances between sites in October for *D. labrax*. Severe drought conditions in 2009 (Instituto de Meteorologia, 2010) resulted in low flow conditions that likely enhanced the establishment of isotopically distinct food webs (Vinagre et al. 2010). It is tempting to speculate that drought events, as a result of decreased productivity and growth (Dolbeth et al. 2008) may delay potential movements within estuaries as well as emigration to coastal environments by marine migrant fish.

Though spatial differences in the isotopic signatures of *D. labrax* and *P. microps* were found, turnover rates, or the speed at which an individual reaches a new isotopic equilibrium following a shift to isotopically distinct prey, are unknown for these species. Larvae generally have very fast turnovers (days) whilst age-0 juveniles are expected to equilibrate within weeks to months (Herzka 2005, Bloomfield et al. 2011). Thus, in July and October, *D. labrax* and *P. microps* had in general been near their collection sites long enough to equilibrate to the local food webs (see França et al. 2011). Overall, tissue stable isotope signatures are particularly valuable to assess small scale movement patterns and identify transient individuals over relatively short time frames (Herzka 2005, Suzuki et al. 2008, Dierking et al. 2012). Future research on turnover rates, including laboratory and field translocation experiments, will be key to enhance our understanding of the temporal accuracy of movement estimates, as well as resolve fish environmental histories via muscle stable isotope signatures (Herzka 2005, Rodgers & Wing 2008).

Analogous to isotopic tissue data, elevated classification accuracies to estuarine sites via otolith elemental composition illustrated that fish comprised geographical groupings within the estuary and that they remained near collection sites shortly before capture (Miller 2007). The



discrete elemental signatures likely result from the exposure to distinctive water and environmental characteristics (see Tanner et al. 2013). Were fish to be moving long distances or recurrently between sites, a decrease in classification accuracies as well as potential overlaps between otolith elemental signatures would have been expected. Misclassifications of a few individuals are probably the result of the natural variability found in otolith composition due to the influence of factors such as individual growth and physiology (Campana 1999, Elsdon et al. 2008).

Otolith chemistry has been used to unravel habitat use in earlier life history periods of fish, than those attainable via muscle tissue isotopic signatures (Verweij et al. 2008). In contrast to tissue stable isotopes, otolith elemental signatures are permanently retained and past environmental signatures can be assessed by analysing the portion of the otolith that corresponds to the life period of interest (Campana 1999). Our analysis targeted the otolith edge (i.e. material laid down shortly before capture) and, given expected daily increment widths (c. 2 to 5  $\mu\text{m}$ ) (e.g. Gutiérrez & Morales-Nin 1986), characterised a shorter and more recent time period prior to fish collection (few days up to two weeks), in comparison to muscle isotopic signatures and expected turnover rates. Overall, both techniques highlighted the low connectivity of *D. labrax* and *P. microps* at their respective temporal scales. Yet, there was an individual of *D. labrax* which had opposing isotopic and otolith elemental scores in the combined plot. Even if there was no overlap in the stable isotopic signatures, or any individual signatures within another site's central isotopic range ( $\pm 1$  ‰, Fry et al 1999), this may be a potential indication of movement: otolith chemistry scores matched the habitat where the fish was collected, and stable isotopes prior habitat use.

Green et al. (2012) found in some cases that *P. microps* from segregated salt marshes had similar isotopic signatures. These were interpreted as a result of similar environmental conditions, rather than indicative of connectivity between sites, due to the distance between them. In this case, the application of further natural tags such as otolith elemental signatures could have complemented isotopic signatures for identifying source sites and fish movements, based on potential variations in water chemistry besides dietary specialization (Verweij et al. 2008, Lawton et al. 2010, Dierking et al. 2012). Lack of differences between fish isotopic signatures may not necessarily denote connectivity, hence the importance of baseline studies acknowledging if food web isotopic patterns vary at spatial scales of interest. The same is applicable for otolith chemistry. If there are no differences between analysed environments we cannot expect differences in natural tags.

Combining otolith chemistry with tissue isotopic signatures on the same individuals provided complementary information on fish habitat use at different temporal scales whilst elucidating distinct ecological and environmental linkages, even if applied to a restricted number of specimens. Classification errors were reduced based on combined data, and the explored graphical approach was complementary to the single natural tag approaches, enhancing the connectivity assessment. The approach is flexible enough to be used with different approaches and to the integration of different natural tags, and can depict individual variability as well as

individuals whose natural tag signatures potentially resemble those from other sites. Overall, combining natural tags shows great promise to disentangle estuarine connectivity patterns (e.g. Verweij et al. 2008, Miller et al. 2010, Perrier et al. 2011).

Ultimately, unravelling connectivity and obtaining a better understanding of habitat use and fish movement patterns within estuaries will have significant ecological and management applications, and will be paramount in defining the spatial scale at which management and conservation strategies are to be developed. Lack of such information can lead to loss or degradation of ecologically important estuarine habitats without a full understanding of their structural and functional significance.

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## CHAPTER 6

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### **Connectivity between estuarine and coastal fish populations: contributions of estuaries are not consistent over time**

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## Connectivity between estuarine and coastal fish populations: contributions of estuaries are not consistent over time

**Abstract:** Otolith elemental signatures (Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca and Pb:Ca) of age-0 juveniles of flounder *Platichthys flesus* and sea bass *Dicentrarchus labrax*, collected from estuarine systems along the Portuguese coast in 2006 and 2009 were used as baseline signatures to identify the estuarine nursery of age-3<sup>+</sup> and 2<sup>+</sup> coastal fish matching the juvenile year classes. Otolith elemental signatures were determined via Laser Ablation Inductively Coupled Plasma Mass Spectrometry, and successfully identified the estuarine origin of the majority of coastal fish to characterized baseline estuaries. Relative contributions of individual estuaries to coastal areas, quantified using maximum likelihood estimation approach, varied over time for the two year classes analysed. Yet, some general patterns were discernible in terms of important estuaries and those with minor or negligible contributions. Assigned nursery origins varied among species and suggested large scale movements along the coast. For *P. flesus* the main source for recruits changed between year classes from Ria de Aveiro (69 %) to the Douro estuary (59 %). The Mondego estuary, located near the latitudinal limit of *P. flesus* occurrence contributed moderate numbers to coastal populations. The Tejo estuary was the most important estuary for *D. labrax* in both years (55 and 50 %), whilst the Ria de Aveiro hardly contributed to sea bass coastal populations (<2 %). Ultimately, understanding the extent to which estuaries replenish coastal adult populations, and fisheries both local and distant, is essential for effective management and conservation of these species and their estuarine juvenile habitats.

**Keywords:** Connectivity; juvenile fish; nursery; otolith chemistry; elemental signatures; Portugal

### 1. Introduction

The ecological and economic importance of estuarine nursery function to the replenishment of coastal fish populations has been widely recognized. Many fishes use estuaries to complete their life cycles, with larvae or early juveniles often spending months to years in these environments before recruiting to coastal adult populations (see reviews by Beck et al. 2001, Able 2005). Thus, juveniles and adults of these species are spatially segregated and it is fundamental to determine the exchange of individuals among these geographically separated groups, i.e. connectivity, which is a key factor in the regulation of population dynamics, colonization patterns and resilience to harvest (Thorrold et al. 2001, Cowen et al. 2007). Assessing fish movement patterns between estuarine habitats and coastal environments, whilst determining the sources of juveniles to adult populations, is increasingly recognized as a vital link to the identification of ecologically important habitats and the development of effective management strategies for coastal fish populations, many of which are commercially exploited (Gillanders 2005, Vasconcelos et al. 2011). However, the spatial scale and movement patterns between estuarine juvenile and coastal adult populations is still poorly understood for many species, and estimates of the effective contributions of juvenile habitats to coastal adult populations is generally lacking (Gillanders 2002, Hamer et al. 2005, Rooker et al. 2010).

Unravelling fish movement and quantifying connectivity is a complex task. Despite considerable progress most artificial tagging techniques are not viable for small juvenile fish.



However, recent advances enable the interpretation of distinct biological features as natural tags, and among them the chemical composition of otoliths has developed into a prominent technique to quantify individual fish movement and connectivity patterns (Elsdon et al. 2008, Gillanders 2009). Otolith chemical signatures have been successful in discriminating natal or nursery origins (e.g. Rooker et al. 2001, Vasconcelos et al. 2007, Clarke et al. 2009) and assessing the differential contributions of juvenile source areas to adult populations (e.g. Thorrold et al. 2001, Gillanders 2005, Vasconcelos et al. 2008, Wells et al. 2012). The use of otolith chemical composition as a natural tag is possible because otoliths are metabolically inert, grow continuously and incorporate trace elements as they accrete (reviewed in Campana 1999, Elsdon et al. 2008). Hence, as otoliths form, an elemental signature is incorporated and due to the intrinsic relation between otolith growth and fish age, the environmental record experienced by adult fish during their juvenile estuarine life period can be determined by analysing the portion of the otolith that corresponds to that specific life period.

Along the Portuguese coast several estuaries are acknowledged for their role as nurseries for flounder *Platichthys flesus* (Linnaeus 1758) and sea bass *Dicentrarchus labrax* (Linnaeus 1758). These marine migrant species are valuable resources in Portuguese fisheries and their use and dependence on estuarine areas as juveniles in this coast is well documented (e.g. Cabral et al. 2007, Martinho et al. 2008, Vasconcelos et al. 2010). In view of the commercial importance of these species, a quantitative understanding of the connectivity patterns between estuaries and coastal populations will provide a valuable framework for the development of appropriate habitat conservation strategies and the sustainable management of local and distant fisheries (Hamer et al. 2005, Hamer et al. 2011). If recruitment is found to be dependent on only one or a few source estuaries, ecologically important habitats with higher degrees of connectivity could be prioritized for management and conservation purposes (Vasconcelos et al. 2011). However, estuarine nursery function and connectivity patterns may be influenced both by species ecological features (e.g. inter-annual fluctuations in larval recruitment to estuarine nursery grounds) or environmental conditions, with the value of nursery areas potentially changing with year class (Kraus & Secor 2005). Most studies have only focused on a single year (but see Hamer et al. 2011) however, if estuarine nurseries are to be effectively protected knowledge on the generality of patterns across multiple year classes of adults is required. Therefore, additional research is paramount to ascertain the estuaries that contribute to replenish coastal populations over time or year classes.

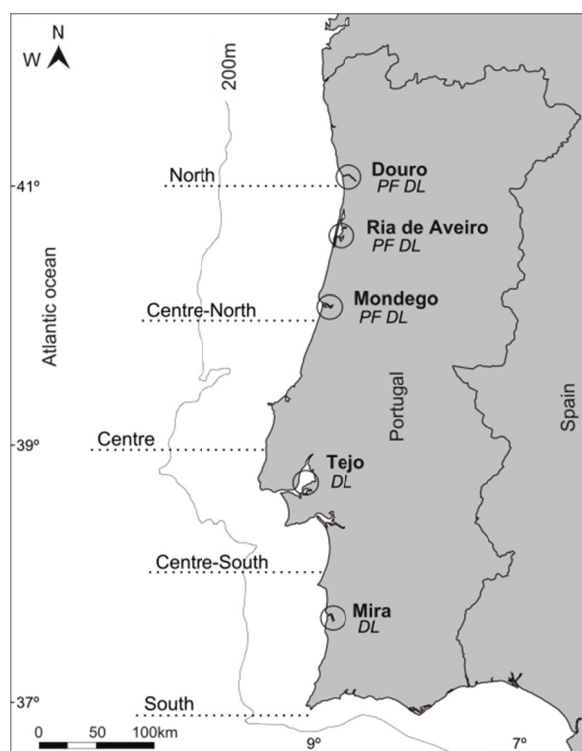
The aim of the present study was to determine the estuarine nursery origin of *P. flesus* and *D. labrax* along the Portuguese coast and quantify the relative contributions of each estuary to coastal populations of these commercially important marine migrant species. Furthermore, this study assessed connectivity in two distinct year classes to evaluate the generality of nursery contributions over time and if the most important source estuaries (i.e. those with higher connectivity estimates) were similar between years. A previous study established the otolith elemental signatures for age-0 juveniles in 2006 and 2009 in the main estuaries where these species occur along the Portuguese coast (Reis-Santos et al. 2012). These estuarine elemental

fingerprints accurately characterized the time period spent by juvenile age-0 fish in estuaries and were used as baseline for the current connectivity assessment.

## 2. Material and methods

### 2.1. Juvenile elemental signatures in estuaries

The baseline multi-elemental otolith signatures used to identify the estuarine nursery of coastal young adult *Platichthys flesus* and *Dicentrarchus labrax* were previously characterized in otoliths of age-0 juveniles sampled throughout 2006 and 2009 in major Portuguese estuarine systems where these species occur (Reis-Santos et al. 2012) (Figure 1). The estuaries in which juveniles were sampled comprise the most likely source-habitats for flounder and sea bass populations along the Portuguese coast (see also Vasconcelos et al. 2011). Species-specific estuarine otolith elemental signatures were previously established for each year with high discrimination among estuaries (overall cross-validated classification accuracy ranged between 77 % and 96 %) (Reis-Santos et al. 2012). Estuarine otolith elemental tags were year specific, which implied that with these natural tags only adults belonging to the 2006 and 2009 year classes could be retrospectively assigned to their estuarine sources (Gillanders 2005, Reis-Santos et al. 2012).



**Figure 1.** Coastal sampling sectors along the Portuguese coast for *Dicentrarchus labrax* (DL) and *Platichthys flesus* (PF). Also shown, location of estuarine nurseries of the two species (open circles) where estuarine otolith elemental signatures of age-0 juveniles were previously characterized (Reis-Santos et al 2012).

## 2.2 Sampling of coastal adult fish

Collection of young adults of *P. flesus* and *D. labrax* was carried out along the Portuguese coast. Specimens were obtained directly from professional fishermen operating locally in five different sectors of the Portuguese coast (Figure 1). Upon collection, fish were transported on ice and dissected at the laboratory. We collected age-3<sup>+</sup> and age-2<sup>+</sup> individuals of both species belonging to the 2006 and 2009 year classes, respectively, based on fish size, in the winters of 2009/2010 and 2011/2012. Sampling focused on fish that approximated the expected fish lengths for the respective age classes, based on established length at age relationships for the Portuguese coast (e.g. Gordo 1989, Teixeira et al. 2010). Ages were confirmed by counts of annual increments on the otolith sections used for chemical analysis.

## 2.3. Otolith preparation and analyses

Otolith sample preparation and analysis closely followed the methodology applied for analyses of the juvenile age-0 fish and the characterization of the baseline estuarine otolith elemental signatures (see Reis-Santos et al. 2012).

Briefly, sagittal otoliths were extracted, using plastic forceps, washed and cleaned of adhering tissue with ultra-pure water and allowed to air dry in microcentrifuge tubes in a positive pressure laminar flow hood. The right otolith of each fish was embedded in EpoFix resin (Struers), spiked with indium (In) at 30 ppm to allow discrimination between otolith material and resin during analysis. Otoliths were sectioned transversely through the nucleus using a low-speed saw (Buehler Isomet). Resulting otolith sections were polished to c. 250 µm with lapping film (30 µm, 9 µm and 3 µm grit size successively, using ultrapure water to remove surface contamination) and mounted on glass slides with indium-spiked thermoplastic glue (CrystalBond 509). Slides were then cleaned, sonicated and triple-rinsed with ultrapure water, dried under a laminar flow hood and stored individually in sealed plastic bags.

A New Wave 213 nm UV high performance (Nd:YAG) laser microprobe coupled to an Agilent 7500cs inductively coupled plasma mass spectrometer (ICP-MS) was used to quantify <sup>7</sup>Li, <sup>24</sup>Mg, <sup>43</sup>Ca, <sup>55</sup>Mn, <sup>65</sup>Cu, <sup>88</sup>Sr, <sup>138</sup>Ba and <sup>208</sup>Pb elemental concentrations in otoliths. Laser ablations occurred inside a sealed chamber with resultant material and sample gas transported to the ICP-MS via a smoothing manifold in an argon and helium stream. We used the same instrument previously used to analyse the otoliths of the age-0 fish. Operating parameters were also identical. Ca was used as an internal standard to correct for variation in ablation yield between samples (Yoshinaga et al. 2000). <sup>115</sup>In was also measured but solely as a marker to discriminate between spiked resin, or CrystalBond, and otolith matrices.

Otoliths were analysed using 300 µm rasters along the increments that corresponded to the juvenile life stage. This corresponded to the otolith section formed while age-0 fish were within estuarine areas and matched the life stage and time period encompassed in the elemental signatures characterized in Reis-Santos et al. (2012). Otoliths were analysed in random order. A certified glass reference standard NIST 612 (National Institute of Standards and Technology) was run at the start and end of each session and after every 10 otoliths to correct for mass bias

and machine drift. External precision (% relative standard deviation) was assessed by measurements of a calcium carbonate certified reference material MACS-3 (United States Geological Survey) and were as follows: 4.9 % (Li), 6.1 % (Mg), 3.3 % (Mn), 5.5 % (Cu), 2.3 % (Sr), 4.2 % (Ba), and 7.8 % (Pb). Data reduction, including background corrections, mass count data conversion to concentrations (ppm) and limits of detection were all performed for each individual sample via Glitter software (GEMOC, Macquarie University, Sydney, Australia).

### 2.3. Data analyses

Otolith elemental concentration data were converted to molar concentrations and standardised to calcium. All further data analyses were carried out on  $\log(x+1)$  transformed element:Ca data. Each species was analysed independently.

Accurate estimations of the relative contributions of estuaries to a mixed coastal stock relies on the assumption that all possible sources that could contribute to that mixed stock have been included in the baseline data set (Campana 1999). Whilst our age-0 juvenile baseline data incorporated a thorough characterization of the otolith elemental signatures of major estuarine nursery areas along the Portuguese coast where these species occur, it is however, possible that other source areas may have contributed to the analysed adult population. These include smaller estuaries or inlets along the Portuguese coast, as well as estuarine sites beyond the Portuguese coast. In these cases, the otolith elemental signatures should not match those of the age-0 baseline data set. Hence, to reduce potential bias of uncharacterized source areas we compared the otolith elemental signatures of adult fish with those of the age-0 juveniles using principal component analysis (PCA) (Hamer et al. 2005, Chittaro et al. 2009). Adult individuals that fell outside a 95 % confidence ellipse around the juvenile baseline data (estuarine elemental signatures) were assumed to have originated from an estuarine nursery area not previously characterized in Reis-Santos et al. (2012), and were excluded from further analyses.

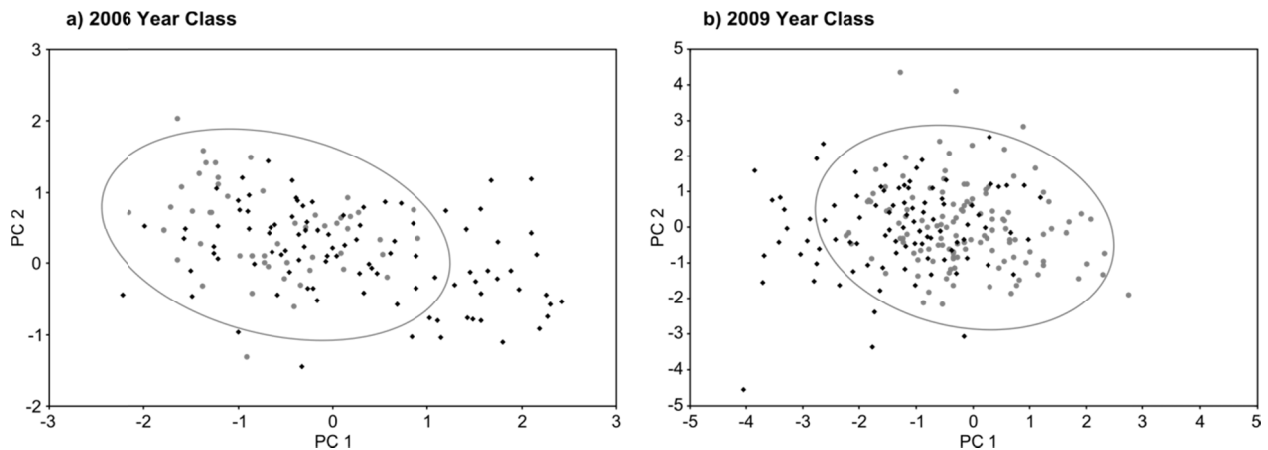
We used a maximum likelihood estimation approach (MLE) (Millar 1987, 1990a) to determine the relative contributions of the different baseline nursery areas to populations of *P. flesus* and *D. labrax* along the Portuguese coast for each year class (2006 and 2009). Proportions of adult origins were also evaluated for each of the five sectors along the coast. Analyses were performed with HISEA (Millar 1990b), generating maximum likelihood estimates and standard deviations of the proportion of the mixed stock (coastal adult populations of each species) originating from the baseline groups. Bootstrapping with a 1000 resampled baseline and adult datasets was used. For each species and year class analysed, baseline data were the multi-elemental otolith signatures of age-0 juveniles characterized in Reis-Santos et al. (2012).

## 3. Results

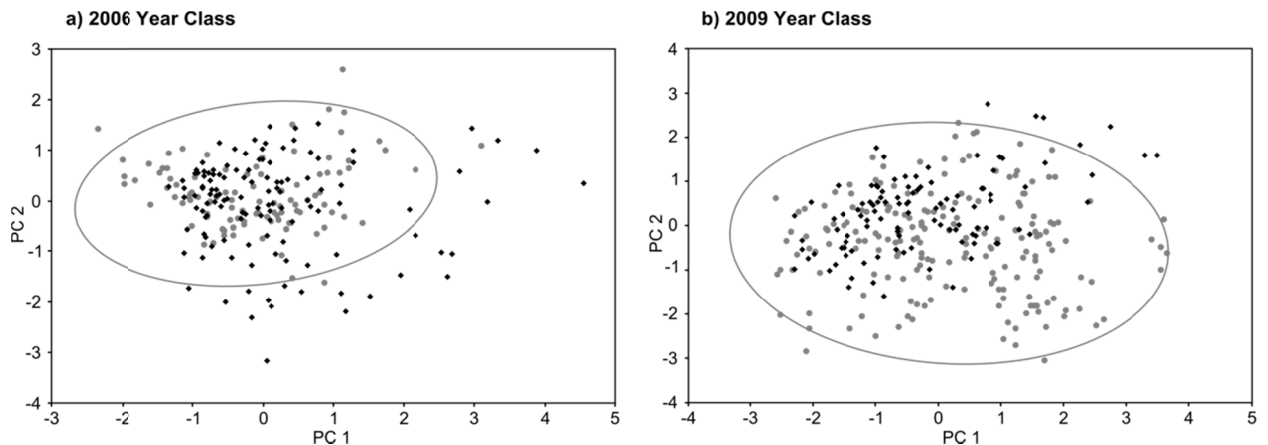
### 3.1. Comparison of adult and juvenile otolith elemental ratios

The elemental ratios from the juvenile section of the otoliths of adult fish generally overlapped the 95 % confidence ellipses of the juvenile baseline data (Figures 2 and 3). Yet, for

both years and species, there were some adult elemental tags that fell outside the confidence ellipses of the juvenile data. We excluded 33 % of adult *P. flesus* (32 individuals; Figure 2a) and 18 % of *D. labrax* (23 individuals; Figure 3a) from the 2006 year class. There was a greater overlap between the otolith chemical composition of adult and juvenile fish belonging to the 2009 year class, with only 23 % of *P. flesus* (21 individuals; Figure 2b) and 6 % (7 individuals; Figure 3b) of *D. labrax* excluded because their elemental signatures fell outside the 95 % confidence ellipses of the juveniles.



**Figure 2.** *Platicthys flesus*. Ordination plot of principal component analysis (PCA) comparing multi-element otolith signatures of adult fish (mixed-stock) (black) and age-0 juveniles of known estuarine origin (baseline group) (grey) for the (a) 2006 and (b) 2009 year class. Ellipses are 95 % confidence ellipses around the baseline group data.



**Figure 3.** *Dicentrarchus labrax*. Ordination plot of principal component analysis (PCA) comparing multi-element otolith signatures of adult fish (mixed-stock) (black) and age-0 juveniles of known estuarine origin (baseline group) (grey) for the (a) 2006 and (b) 2009 year class. Ellipses are 95 % confidence ellipses around the baseline group data.

### 3.2. Variability in contributions of estuarine nurseries

The MLE quantification of the relative contributions of the different estuarine nursery areas to coastal populations along the Portuguese coast varied by species and over time between year classes (Table 1).

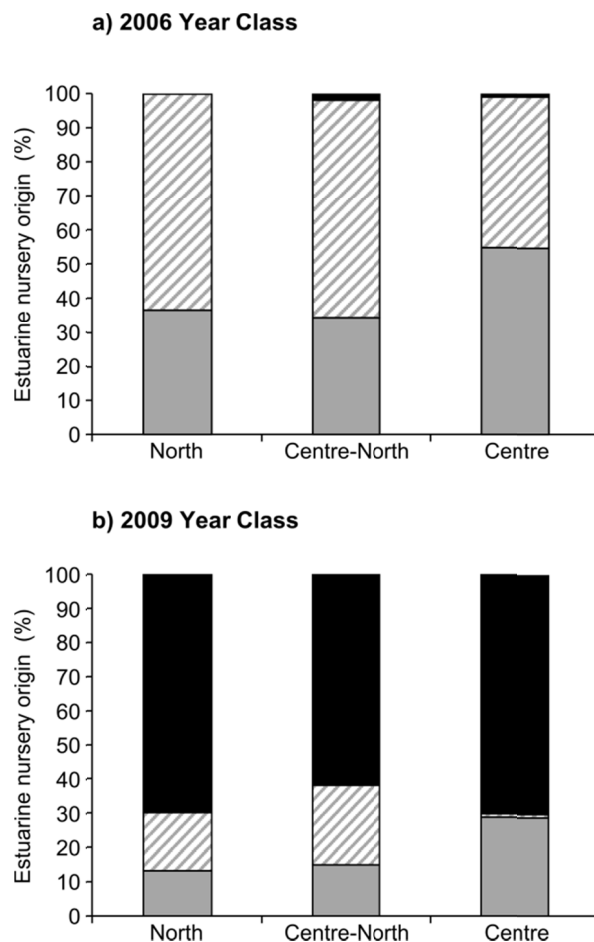
**Table 1.** Maximum likelihood estimates of the relative contributions (%) of estuaries (Douro, Ria de Aveiro, Mondego, Tejo and Mira) to coastal populations of *Platichthys flesus* and *Dicentrarchus labrax* along the Portuguese coast for two distinct year classes. Otolith elemental signatures of age-0 juveniles collected in estuarine nurseries along the Portuguese coast in 2006 and 2009 (previously characterized in Reis-Santos et al. 2012) were used as baseline data to identify the source estuary of coastal adult and sub-adult fish (mixed-stock) of ages-3<sup>+</sup> and 2<sup>+</sup> that matched the 2006 and 2009 baseline year classes, respectively. Mean (and standard deviations) and total number (n) of adults are shown.

Species	Relative contributions (%) of predicted estuarine nurseries of origin					n
<i>P. flesus</i>	Douro	Ria de Aveiro	Mondego			
2006 Year class	3 (2)	69 (8)	28 (8)			65
2009 Year Class	59 (7)	26 (4)	15 (5)			72
<i>D. labrax</i>	Douro	Ria de Aveiro	Mondego	Tejo	Mira	
2006 Year class	8 (3)	1 (1)	39 (6)	50 (7)	2 (1)	99
2009 Year Class	19 (4)	2 (2)	16 (4)	55 (5)	7 (3)	100

The Ria de Aveiro estuary contributed the most (69 %) to the mixed population of *P. flesus* for the 2006 year class, whilst the Douro contributed the least (3 %). In contrast, for the 2009 year class, the largest proportion of adult flounder along the Portuguese coast was from the Douro estuary (59 %), with the Ria de Aveiro contributing only 26 %. The contributions of the Mondego estuary to the mixed stock ranged from 15 % to 28 % in the two year classes.

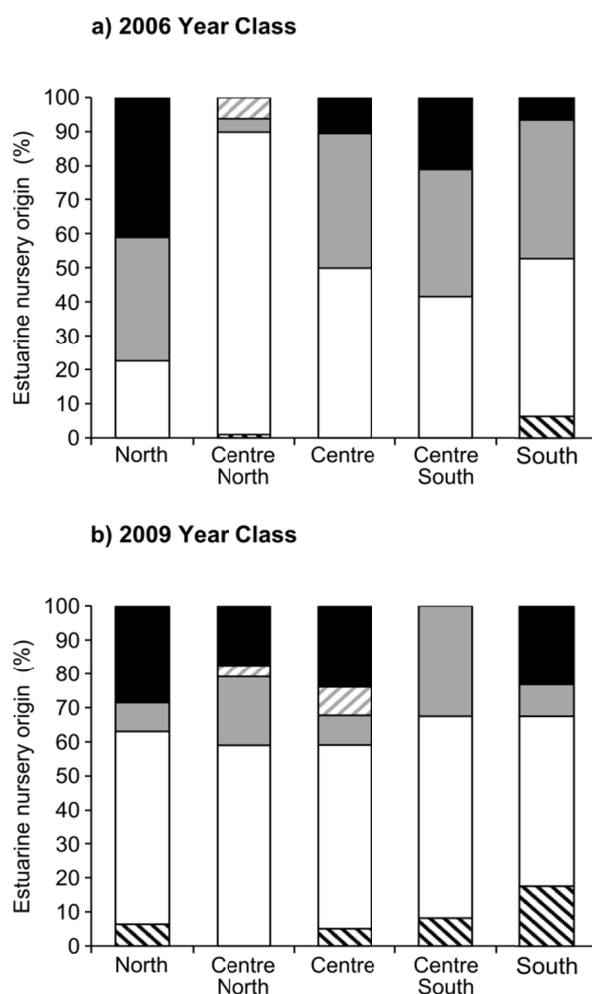
For the 2006 year class, when the different sections of the coast were individually analysed, the Ria de Aveiro contributed with most *P. flesus* to the northern and northern-central sectors, 63 % and 65 %, respectively (Figure 4a). For the central sector of the coast, where this species latitudinal limit of distribution occurs, the majority of fish were identified as having used the Mondego estuary as nursery (55 %). The contributions of the Douro were negligible. For the 2009 year class, 29 % of the flounder from the central sector were also assigned to the Mondego, with contributions to the remaining sectors decreasing to c.15 % (Figure 4b). Estimates for the Ria de Aveiro ranged from 1 % (Centre) up to 23 % (Centre-North), whilst the Douro estuary contributed with the majority of *P. flesus* to each sector.

The Tejo estuary made the highest nursery contribution to the adult population of *D. labrax*, with contributions over 50 % for both year classes (Table 1). The contribution of the Mondego estuary varied by year class (39 % for the 2006 year class compared to 16% for the 2009 year class). Contribution from the Douro estuary increased from 8 % for the 2006 year class to 19 % for the 2009 year class. The Mira, the southernmost estuary, made only small contributions to coastal *D. labrax*, with a maximum of 7 % for the 2009 year class. The Ria de Aveiro was of minor significance for this species with contributions under 2 % for both year classes.



**Figure 4.** *Platichthys flesus*. Maximum likelihood estimates of the relative contributions (%) of estuaries to adult fish caught in each of five sectors along the Portuguese coast for the (a) 2006 and (b) 2009 year classes. From top to bottom, represented estuaries are: Douro (black), Ria de Aveiro (grey lines), Mondego (grey).

Observing the contributions to each sector of the coast individually for the 2006 year class, the Tejo estuary contributed between 23 % (North) and 89 % (Centre-North) of *D. labrax* individuals (Figure 5a). The Tejo and Mondego were the only estuaries to contribute individuals to all the sectors along the coast. Contributions from the Douro and the Mira estuaries were higher in the vicinity of these estuaries. For example, the Douro estuary contributed 41 % of coastal fish in the northern section compared to 0 to 21 % for the other sections along the coastline; and the Mira contributed 7 % of coastal fish to the southern sector, whilst contributions to the remaining sectors were lacking or below 1 % (Centre-North). The same general patterns were found for the 2009 year class (Figure 5b). The Douro, Mondego and Tejo estuaries were the source estuaries for over 80 % of adult *D. labrax* from each section of the coastline. Only the Tejo and Mondego contributed to all five sections of the coast, with Tejo contributions dominating (50 to 60 %), whilst those of the Mondego ranged between 8 % (Centre) and 33 % (Centre-South). Once again, the highest contributions for the Mira estuary were to the local southern sector (18 %); although in the 2009 year class the Mira estuary contributed to all sections of the coast except to the central-northern sector.



**Figure 5.** *Dicentrarchus labrax*. Maximum likelihood estimates of the relative contributions (%) of estuaries to adult fish caught in each of five sectors along the Portuguese coast for the (a) 2006 and (b) 2009 year classes. From top to bottom, represented estuaries are: Douro (black), Ria de Aveiro (grey lines), Mondego (grey), Tejo (white) and Mira (black lines).

#### 4. Discussion

Understanding patterns of connectivity between juvenile estuarine nurseries and coastal adult populations has significant implications for fisheries management and habitat conservation, as it indicates the extent to which populations are replenished. In this study, we were able to quantify the relative contributions of individual estuaries along the Portuguese coast to the coastal populations of *P. flesus* and *D. labrax*. Results highlighted that contributions varied over time for the two year classes analysed. Yet some general patterns were discernible in terms of both important estuaries and those making minor or negligible contributions. Overall, populations along the coast were well mixed, with widespread fish movements occurring.

To avoid confounding effects of inter-annual variations in otolith elemental signatures and consequent bias in the quantification of the relative contributions among source estuaries, we ensured that we matched adult fish to their corresponding age-0 year class (Gillanders 2005, Reis-Santos et al. 2012). Still, a number of coastal fish were removed from the analyses because their otolith elemental signatures (measured in the otolith area matching the juvenile



life period spent within estuaries) did not overlap those of the baseline data (measured in age-0 juveniles). It is expected that movement and dispersal range increase with fish age (e.g. Hamer et al. 2011), thus age-3<sup>+</sup> individuals (year class 2006) upon leaving an estuary potentially disperse further away in comparison to age-2<sup>+</sup> fish (year class 2009). This suggests the higher number of adults from uncharacterized sources in the 2006 year class were likely due to increased numbers of stray individuals from estuaries outside the study area, for example, from sites along the neighbouring Spanish coast. In fact, tagging studies have highlighted complex dispersal and large scale movement patterns for adult sea bass (Pickett et al. 2004, Pita & Freire 2011) and plaice (Dunn & Pawson 2002), a flatfish phylogenetically close to flounder. Moreover, population structure of *D. labrax* and *P. flesus* based on molecular genetic approaches has been shown to be lacking in the North-Eastern Atlantic (Borsa et al. 1997, Naciri et al. 1999, Coscia & Mariani 2011). Overall, the majority of age-2<sup>+</sup> and 3<sup>+</sup> adult sea bass and flounder collected along the Portuguese coast spent their juvenile nursery period within characterized estuaries.

Nursery origins varied among species, and even if many coastal fish were assigned to estuaries in the vicinity of where they were caught (e.g. higher proportion of *D. labrax* from the Mira estuary in the southern sector, or *P. flesus* from the Mondego in the central sector) contributions suggested wide range movements. Such movements are in agreement with described movement patterns and homogeneity of coastal population structure for both species (e.g. Borsa et al. 1997, Pickett et al. 2004, but see also above). As older fish can potentially move further from their recruitment estuaries, it would be of great interest to run tagging or telemetry studies to link with data acquired via otolith natural tags. Moreover, as these fish are long lived, following year classes through time, analysing a wide range of adult year classes and increasing the sampling area to investigate contributions to distant coastal locations (Hamer et al. 2005, Rooker et al. 2010, Hamer et al. 2011) could also aid in resolving the unidentified coastal spawning grounds for these species along this coast.

Relative contributions of individual estuarine nurseries to coastal populations varied over the years for analysed age classes. The striking increase in contributions from the Douro estuary for *P. flesus*, from least to most important, was noteworthy and was accompanied by a decrease of contributions from the Ria de Aveiro, even though the latter still maintained a moderate level of contributions for the 2009 year class. Overall, particular estuarine nurseries showed consistently higher contributions, whilst others were negligible (e.g. Tejo and Ria de Aveiro for *D. labrax*, respectively). Very few analogous studies have addressed connectivity contributions of source areas over multiple years, with variations over time reported (e.g. Hamer et al. 2005, Kraus & Secor 2005, Chittaro et al. 2009). Comparisons of the present data to a preliminary single year assessment for these species (Vasconcelos et al. 2008), further outlined annual differences in estuarine relative contributions. However, the use of distinct analytical procedures to determine baseline and adult signatures in that study may be in part responsible for the observed differences between contribution estimates.

Major contributions determined for the Tejo and Mondego estuaries for the 2006 *D. labrax*

year class, are in agreement with the assumption that higher estuarine juvenile densities will account for increased recruitment to coastal populations (e.g. Martinho et al. 2008, Vasconcelos et al. 2010). However, that was not the general case, with inconsistent observations between the importance of estuaries to coastal stocks and reported juvenile densities within the estuaries. For instance, the minor contribution of the Douro estuary to coastal populations of the 2006 *P. flesus* year class did not relate to juvenile densities; reported as highest in this system (Vasconcelos et al. 2010). Overall, assessments combining potential nursery value (e.g. measuring density, growth, available habitat) and effective contributions (e.g. via otolith elemental signatures) have had mixed results depending on species (Vasconcelos et al. 2011); strong matches (Fodrie & Levin 2008); and contributions not explained by variability in juvenile densities (Chittaro et al. 2009). Still, these assessments do not specifically answer why differential connectivity rates are observed. Environmental and biotic factors controlling juvenile survival/mortality rates and migration could be occurring, and influencing recruitment to coastal populations (Levin & Stunz 2005). Reduced growth through intraspecific competition in high density areas, or increased predation pressures due to increased predator aggregations may also explain the lack of relation between densities and connectivity (Laurel et al. 2007, Temming et al. 2007). Further studies are needed to determine the link between connectivity measures and underlying processes.

Assessing the connectivity patterns of *P. flesus* is of particular interest as this species limit of distribution occurs along the Portuguese coast (central sector), with the Mondego the southernmost estuary where juveniles can now be found (Vasconcelos et al. 2010). This species southern limit of distribution has been receding North, and only two decades ago juvenile *P. flesus* were abundant in the Tejo estuary (Cabral et al. 2001). Populations living at the border of the distribution range are expected to live near the limits of their physiological tolerance, and so are more vulnerable to changes in abiotic conditions, with adaptive responses to thermal and anthropogenic stress already suggested for the southernmost populations of *P. flesus* (Calvés 2011). In this scenario, changes in population trends may occur at small spatial and temporal scales, and although estuaries further north attained higher contributions, the Mondego estuary still played a role in the replenishment of the coastal areas along the Portuguese coast for both year classes.

Overall, little information is available on the dispersal of early life stages (eggs and larvae) of *P. flesus* and *D. labrax*, as well as on the rate and spatial extent of adult movements. Measuring connectivity, particularly on fish with segregated life histories, we should be prepared to find out that it may not be identical at all life stages (e.g. Tobin et al. 2010). Hence, future research should focus on investigating adult movement, spawning areas and larval dispersal for these species, in an attempt to ascertain in which life stage connectivity is maximized.

Complex life cycles and segregated habitats have key implications in the development of management strategies, including protected areas, as these will have to address both conservation of juvenile habitats as well as the sustainable management of coastal adult populations (Kraus & Secor 2005, Di Franco et al. 2012). Priority should be given to habitats

with consistently higher connectivity and contribution rates, in particular if they act as source sites for multiple species simultaneously (Gillanders 2002, Vasconcelos et al. 2011). Nonetheless, the temporal variability in connectivity patterns, regarding changes in the relative importance of estuaries to coastal populations, suggests that safeguarding estuaries where moderate or elevated contributions occur irregularly may act as a buffer to dampen variation in year class strength to coastal populations (Rooper et al. 2003, Chittaro et al. 2009). Ultimately, widespread anthropogenic pressures threaten juvenile estuarine habitats, thus understanding the extent to which estuaries replenish coastal adult population, and fisheries both local and distant, is essential for an effective management and conservation of these species and their nursery habitats.

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## CHAPTER 7

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General discussion

Final remarks

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## **General discussion**

### **Future perspectives**

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#### **General discussion**

The present study capitalized on the natural tag properties of otoliths to determine connectivity patterns between estuarine and coastal systems and if these changed over two distinct years. In addition, specific aspects regarding the reliability and effective application of otolith elemental signatures to assess connectivity, reconstruct fish movement patterns, habitat use and environmental histories were explored. Overall, results highlighted the value and efficacy of otolith elemental composition to assess connectivity and provided much needed information to implement and evaluate spatial management and protection measures. Understanding population connectivity is essential to devise appropriate management scales and establish spatially explicit harvesting strategies, whose measures should ideally be dictated by the dispersal and connectivity of multiple key species.

Estuaries are complex ecosystems, supporting fundamental ecological links with other environments through highly interconnected processes among their physical, chemical and biological components (Beck et al. 2001, Able 2005). The comprehensive literature review in Chapter 2 provided the starting point and conceptual background for this study as it outlined research gaps and potential frameworks to progress our understanding of connectivity, with particular emphasis on the use of otolith elemental signatures as natural tags; which were subsequently examined in the following chapters. Focus was directed towards estimating successful movement of juveniles from estuaries to coastal areas, and quantifying their effective contribution to adult populations over time.

The ability of otolith chemistry to address numerous goals in ecology and fisheries science has resulted in its widespread application. However, many uncertainties still linger regarding the factors affecting elemental uptake and incorporation in otoliths, which, in particular, limit the reconstruction of fishes' environmental histories (Elsdon et al. 2008, Sturrock et al. 2012). The interpretation of otolith chemical patterns and their relationship with environmental parameters can be validated via experimental trials. In Chapter 3, temperature, salinity and water chemistry variations were examined to evaluate their interactive effects on otolith chemistry and elucidate its potential to interpret field-derived data. Consistent interactions between salinity and water elemental concentration were found for both Sr:Ca and Ba:Ca, as well as positive effects of temperature on elemental composition and incorporation. Determining the degree to which movements across expected environmental gradients can be confidently inferred via otolith chemistry is a critical step before wild caught fish can be effectively linked to their environmental history (Elsdon et al. 2008, Hicks et al. 2010, Macdonald & Crook 2010). Present findings are



the starting point to unravel environmental reconstructions, and could be valuable to illustrate habitat use patterns and identify essential habitats for the marine migrant fish *D. labrax*. Nonetheless, it will be important to evaluate the consistency of laboratory trials versus field collected fish to establish the generality of experimental data on otolith chemistry (Elsdon & Gillanders 2005, Dorval et al. 2007, Elsdon et al. 2008). This will be particularly interesting and challenging in estuaries, as it is likely that physicochemical characteristics of estuarine water masses change across short spatio-temporal scales (in addition to other factors such as food availability, diet, oxygen or photoperiod which are constant in laboratory trials), which may lead to divergences between laboratory and field studies (Dorval et al. 2007). Nonetheless, otolith chemistry has been linked to estuarine ambient concentrations, and specific elements acknowledged as valuable tracers of habitat use (Wells et al. 2003, Elsdon & Gillanders 2005, Tanner et al. 2013). Considering the complexity of estuarine environments, and the interactive effects of environmental parameters on otolith chemistry (Chapter 3), it may be necessary to examine multiple markers to provide an appropriate framework to reconstruct habitat use and environmental histories in euryhaline fish. On the other hand, understanding the complex mechanisms underlying otolith elemental incorporation is not required to use otolith elemental signatures as group specific natural tags (Thorrold et al. 1998, Elsdon et al. 2008).

The spatio-temporal variability of otolith elemental signatures in estuaries was investigated in Chapter 4. Confirming if elemental tags vary at spatial scales of interest, together with an evaluation of their temporal changes, are the first steps in any analysis of otolith chemistry and a prerequisite to apply otolith elemental signatures in connectivity assessments (Campana et al. 2000, Elsdon et al. 2008). Elemental tags may vary over different spatial scales (Hamer et al. 2003, Rooker et al. 2003, Miller 2007, Clarke et al. 2009, Chapter 4), with the diversity of environmental conditions in estuaries particularly suited to the occurrence of fine scale spatial discriminations (e.g. Miller 2007, Chapter 5). Similarly temporal variation can be found at different scales, from weeks to months and years (see Hamer et al. 2003, Walther & Thorrold 2009, Tanner et al. 2013, Chapter 4), and is particularly important for the retrospective determination of sites of origin as temporal variations may confound spatial discrimination and lead to incorrect estimates of connectivity. Estuarine elemental signatures of *P. flesus* and *D. labrax* effectively distinguished among nursery estuaries yet showed seasonal and annual variations. One of the objectives of this study was to establish robust baseline elemental fingerprints to later identify the nursery estuary of adult fish caught off the coast (Chapter 6). Since estuarine otolith elemental tags were found to be year specific, elemental fingerprints used to retrospectively classify coastal adults have to be from juveniles of the same year classes of adult fish (Gillanders 2002). However, monthly variations resulted in negligible differences in the accuracy of estuarine discrimination; therefore elemental signatures were established incorporating seasonal variation to establish elemental signatures that were representative of a juvenile year class. This is a great advantage for connectivity assessments, and reduces the likelihood of errors matching juvenile and adult signatures. Even so, very few studies have used individuals collected over monthly periods to characterize baseline elemental

signatures (Thorrold et al. 1998, Hamer et al. 2003). Promoting processes that cover spatio-temporal variability in natural tags and take into account individuals' life histories are essential (Cook 2011). In this case, pooled seasonal tags were representative of a year class and encompass the main period juveniles remain within estuaries.

Whether fish move among segregated estuarine habitats, or show high site fidelity, has major ecological and management implications. Ultimately, anthropogenic pressures in estuaries, conspicuous worldwide, may hinder the replenishment of coastal populations if ecologically important habitats for juvenile fish are lost (Kennish 2002, Vasconcelos et al. 2007) or habitats become more fragmented. Describing movement and habitat use patterns is fundamental to identify essential fish habitats and define the spatial scales at which to devise conservation measures. This is of particular interest since marine migrant species often occur in distinct sites or habitats within an estuary before recruiting to coastal stocks (e.g. Martinho et al. 2007, Vasconcelos et al. 2010). Therefore, a combined application of otolith elemental signatures and soft tissue isotopic signatures addressed the movement and habitat use patterns of juvenile *D. labrax* and *P. microps* between segregated estuarine sites (Chapter 5). Overall, estuarine habitat use patterns are a result of fish responses to multiple environmental variables and influenced by behavioural and physical processes that act over a range of spatio-temporal scales (Stoner et al. 2001, Bacheler et al. 2009, Vasconcelos et al. 2010). In this instance, the combined use of both natural tags exposed distinct ecological and environmental linkages, and enhanced the connectivity assessment as both techniques provide independent, but complementary, information at distinct scales. Yet, approaches combining these natural tags are still scarce (Verweij et al. 2008, Lawton et al. 2010, Dierking et al. 2012). *P. microps* was assessed concurrently to *D. labrax* as it was hypothesised it would be a valuable proxy species of limited home range (Cattrijse & Hampel 2006, Dierking et al. 2012) and could be used as a reference to evaluate changes in spatial variations of isotopic and elemental signatures. Over the study period, connectivity among the segregated estuarine habitats was low (akin to Vinagre et al. 2008, Green et al. 2012). In this context, localized disturbances, diminishing habitat quality and food web structure among others, can likely affect growth and vital rates of fish in estuaries, undermining the potential yield of nursery areas and their contributions to coastal stocks.

The relative contributions of estuaries to the coastal populations of *P. flesus* and *D. labrax* over two distinct years was successfully estimated (Chapter 6). The successful classification to individual estuaries, using the baseline elemental signatures characterised beforehand (Chapter 4), provides the basis for further investigations at this estuarine scale along the Atlantic and Mediterranean coasts. The geographical range of these studies should ultimately match species potential dispersal scale. In the present assessment, the majority of coastal adult fish spent their juvenile nursery period in one of the estuaries included in the baseline assessment, with widespread fish movements occurring for both year classes. Yet, alternative nursery sources for flounder or sea bass may exist along the Portuguese or neighbouring coasts. Though changing over time, a restricted number of estuaries were responsible for larger contributions to adult coastal populations. Very few studies have so far addressed variations in connectivity patterns

over time (Hamer et al. 2005, Kraus & Secor 2005, Chittaro et al. 2009, Hamer et al. 2011) but single measures of connectivity will seldom be sufficient to describe the dynamic interactions between juvenile estuarine and adult coastal populations. For example, inter-annual fluctuations in larval recruitment to estuaries, or processes occurring locally within estuarine nurseries regulating year class strength (Gibson 1994, van der Veer et al. 2000, Le Pape et al. 2007, Martino & Houde 2010) may lead to variation among years (Chapter 6). Thus, to invest conservation funds wisely, it is fundamental to ascertain the generality of connectivity patterns for key species and prioritize ecologically important sites (Beck et al. 2001, Dahlgren et al. 2006), i.e. those that most consistently replenish coastal populations over time, as this will provide the greatest returns in protected resources and support the delineation of effective management and conservation strategies (Gillanders et al. 2011, Vasconcelos et al. 2011). Besides connectivity in different year classes, examining spatial variations in connectivity throughout a fish's lifetime by following a year class over time is recommended (see also Hamer et al. 2011). This could enable the detection of age-related movement patterns, such as those towards spawning grounds. As larger older adults may have broader migration potentials, fisheries management must consider the potential loss of connectivity due to overfishing. Overall, current results for the two analysed year classes suggest coastal populations along the Portuguese coast were well mixed. For *D. labrax*, artificial tagging studies have documented adult fish movements over hundreds of kilometres (Pickett et al. 2004, Pita & Freire 2011), the same capability would be expected for *P. flesus* considering movement in other flatfish such as plaice or sole (Koutsikopoulos et al. 1995, Dunn & Pawson 2002). On the contrary, remarkably little is known regarding spawning grounds and dispersal of early life history stages (i.e. eggs and larvae) of these species, in particular along the Portuguese or nearby coasts.

Despite recent evidence that local retention may be more likely than previously assumed (Swearer et al. 2002, Almany et al. 2007) early life stages may be transported to distant locations before settlement into estuarine nursery grounds. The rate and spatial extent of connectivity may vary among life history stages (DiBacco et al. 2006, Tobin et al. 2010); therefore future research should focus on adult movement, spawning areas and larval dispersal for these commercially important species, to ascertain in which life stage connectivity is maximized, and further support practical efforts to implement habitat conservation and integrated fisheries management directives. It is important that nursery areas or spawning aggregations are protected as this will enhance fisheries yield. Ultimately, managing these species with an understanding of spatial structure and connectivity is an increasingly important tool in safeguarding their persistence and promoting coastal fisheries sustainability.

### **Future perspectives**

Upon successful evaluations of fish connectivity using otolith chemistry and progress in determining and incorporating temporal variability in otolith elemental signatures; unravelling how environmental factors affect otolith elemental incorporation; and integrating distinct natural markers to enhance habitat use assessments, future research should focus several issues to

further contribute to our understanding of connectivity among fish populations. Given the presence of species specific effects on otolith chemistry (Gillanders & Kingsford 2003, Reis-Santos et al. 2008, Melancon et al. 2009) the development of a universal proxy for elemental incorporation and uptake is unlikely. Nonetheless, additional studies in laboratory and field settings (e.g. field caging studies, or combined applications of natural and artificial tagging) on multiple species should further resolve why elemental signatures differ, and ascertain the physiological and genetic regulation of element incorporation in otoliths. Such research will help establish predictable links between otolith chemistry and environmental parameters (Campana 1999, Elsdon et al. 2008, Sturrock et al. 2012).

Connectivity assessments should be expanded to wide scale evaluations (e.g. Walther & Thorrold 2008), and upcoming investigations on connectivity patterns should match the organism's dispersal scale and encompass its different life stages. The use of multiple and potentially complementary techniques that integrate information over different spatio-temporal resolutions likely provides the best inference on fish population structure and connectivity (Thorrold et al. 2002, Hedgecock et al. 2007, Woods et al. 2010). The hierarchical integration of genetic markers (e.g. DNA microsatellites) with otolith chemistry is an important step towards disentangling population structure and connectivity of many commercially important species (Miller et al. 2005, Bradbury et al. 2008a, Barnett-Johnson et al. 2010, Miller et al. 2010, Svedäng et al. 2010), namely for *P. flesus* and *D. labrax*. Genetic markers will provide insight on gene flow at recent or evolutionary time scales, whilst otolith chemistry excels over single or a few generations. Additionally, information from artificial tags may be particularly useful to further clarify home range and habitat use (e.g. Pawson et al. 2007, Bradbury et al. 2008b, Abecasis et al. 2009, Pita & Freire 2011). Overall, these could support the delineation of the appropriate spatial and temporal scales for management and conservation measures.

Examining the underlying ecological processes that influence connectivity (e.g. growth, density, abundance, survival) (Chapter 1, van der Veer et al. 2000, Fodrie et al. 2009, Vasconcelos et al. 2011) and directly evaluating recruitment variability as a function of processes affecting larval and juvenile life stages, in particular regarding those modifying estuarine nursery function, are a key issue towards disentangling why higher connectivity is observed in particular sites or habitats. One of the greatest challenges would also be to integrate coupled biophysical models to contribute to our understanding of larval dispersal and sources of variability in recruitment (Santos et al. 2007, Werner et al. 2007, Cowen & Sponaugle 2009, Petitgas et al. 2013). Ultimately, scenario based modelling could be used to predict how environmental changes and distinct anthropogenic pressures affect connectivity, as well as the implications or effectiveness of implementing distinct management and conservation strategies (e.g. protected areas, spatially-explicit harvesting strategies). Model applications will be paramount to a conceptual understanding of how species persist in seascapes modified by human activities and highlight where management and conservation efforts can be invested more wisely towards greater returns in the long term.

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