Universidade de Aveiro Departamento de Biologia
Ano 2017

FERNANDO ANTÓNIO FRANCISCO RICARDO

UTILIZAÇÃO DE FERRAMENTAS BIOGEOQUÍMICAS NA RASTREABILIDADE DE ORIGEM DE BIVALVES – PRIMEIROS PASSOS PARA A CERTIFICAÇÃO DE ORIGEM

USE OF BIOGEOCHEMICAL TOOLS TO TRACE THE ORIGIN OF BIVALVES – FIRST STEPS TOWARDS ORIGIN CERTIFICATION

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia Marinha, realizada sob a orientação científica do Doutor Ricardo Jorge Guerra Calado, Investigador principal do Departamento de Biologia da Universidade de Aveiro e sob a co-orientação científica da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora associada com agregação do Departamento de Química da Universidade de Aveiro

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Dedico este trabalho ao Tomás e Afonso, meus filhos, e à Marta, minha esposa.

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palavras-chave *Cerastoderma edule*, produtos alinebtares de origem marinha, origem geográfica

resumo

A globalização da indústria de comercialização de produtos alimentares e os recorrentes alertas sobre questões de segurança alimentar, resultaram numa crescente consciencialização dos consumidores sobre a necessidade de rastrear estes produtos. Determinar a origem geográfica de produtos alimentares de origem marinha é fundamental para controlar a sua qualidade e salvaguardar o interesse dos consumidores. Este estudo utilizou como espécie alvo o berbigão (*Cerastoderma edule*), focando-se na utilização de ferramentas bioquímicas e geoquímicas, tais como perfis de ácidos gordos e a assinatura elementar de conchas, respetivamente, para a determinação da origem geográfica. Esta espécie de bivalve representa uma grande importância comercial e suporta uma série de pescarias nas águas costeiras europeias e, no caso particular da Ria de Aveiro, uma lagoa localizada na costa ocidental atlântica de Portugal, onde a apanha de berbigão é superior a 1000 toneladas por ano.

O primeiro passo do presente estudo consistiu na avaliação do potencial uso dos perfis de ácidos gordos do músculo adutor de *C. edule* comercializados frescos para rastrear seu local de origem. Os resultados mostraram, pela primeira vez, que é possível determinar a origem geográfica com resolução espacial < 10 km sem o uso complementar de assinaturas de isótopos estáveis. Além disso, o perfil de ácidos gordos do músculo adutor de berbigão fresco mostrou ser capaz de discriminar a origem dos espécimes recolhidos em áreas próximas com diferentes classificações de acordo com o Regulamento Europeu (CE) n. º 1379/2013 para a captura/produção de bivalves. Esta abordagem é primordial para a rastreabilidade, de modo a combater a fraude e a segurança alimentar. Os perfis de ácidos gordos do músculo adutor *de C. edule* foram avaliados ao longo da costa Portuguesa, bem como a sua variabilidade inter-anual dentro do mesmo ecossistema. Os resultados obtidos permitiram diferenciar os berbigões produzidos em diferentes ecossistemas ao longo a costa portuguesa, desempenhando um papel fundamental para os mariscadores/produtores dispostos a diferenciar e agregar valor aos seus produtos. Além disso, esta abordagem foi capaz de discriminar os ecossistemas que são microbiologicamente mais seguros. Os perfis de ácidos gordos do músculo adutor apresentaram variabilidade inter-anual devendo ser considerada para a rastreabilidade, na medida em que compromete a discriminação da origem geográfica. Numa outra tentativa de garantir a segurança alimentar, este estudo determinou se o perfil de ácidos gordos do músculo adutor de berbigão vivo apresentava mudanças significativas durante o tempo de prateleira (sete dias pós-colheita em ambiente refrigerado) e quanto tempo pós-colheita esses perfis de ácidos gordos poderiam ser utilizados para rastrear com fiabilidade a sua origem geográfica. Os resultados indicaram que os perfis de ácidos gordos permanecem estáveis até ao terceiro dia pós-colheita, podendo ser usados para rastrear com fiabilidade a origem geográfica. Após este período, os berbigões começaram a exibir perfis contrastantes de ácidos gordos no seu músculo adutor, nomeadamente uma elevada percentagem do ácido heptadecanóico (17:0), associado ao crescimento de microrganismos patogénicos responsáveis pela deterioração dos alimentos.

Neste estudo foi também avaliada e validada a eficiência da assinatura elementar das conchas de bivalves frescos como um proxy para discriminar a origem de espécimes coletados em áreas adjacentes no mesmo sistema estuarino, tendo sido quantificados Bário (Ba), manganês (Mn), magnésio (Mg), estrôncio (Sr) e chumbo (Pb). Os resultados mostraram, pela primeira vez, que este método pode ser utilizado para obter uma certificação confiável e precisa da origem para bivalves com resolução espacial < 1 km. A análise elementar também foi abordada no sentido de avaliar se a assinatura elementar das conchas de espécimes capturados em oito ecossistemas diferentes ao longo da costa atlântica portuguesa pode ser usado para discriminar com sucesso a sua origem geográfica. Além disso, foi também testado se a assinatura elementar das conchas se altera em dois anos consecutivos, em áreas com classificações diferentes dentro do mesmo ecossistema. A assinatura elementar exibida pelas conchas de berbigão determinou com sucesso a origem geográfica dos berbigões ao longo da costa portuguesa, necessitando, no entanto, de uma verificação periódica (> 6 meses e < 1 ano) para controlar a variabilidade temporal sempre que comparados espécimes provenientes da mesma área recolhidos com mais de seis meses de diferença. As ferramentas moleculares desenvolvidas durante este estudo representam um benefício económico se e quando aplicadas ao setor de produção de bivalves. A transferência desta tecnologia para a produção de bivalves constitui uma forma de segurança do produto, promoção e diferenciação, bem como uma ferramenta de combate à fraude.

keywords *Cerastoderma edule*, seafood, geographic origin

abstract Market globalization and recurrent alerts on food safety issues resulted in a growing awareness of consumers on the need for food traceability. Determining seafood geographic origin is critical for controlling its quality and safeguarding the interest of consumers. This study used as target species the common cockle (*Cerastoderma edule*) and focused in the use of biochemical and geochemical tools, like fatty acid (FA) profiles and shells' trace element fingerprints (TEF), respectively, to determine the geographic origin. This bivalve species represents a high commercial importance and supports a number of fisheries in European coastal waters and, in the particular case of Ria de Aveiro, a lagoon located in the western Atlantic coastal of Portugal, the harvesting of cockle exceeds 1000 tons per year.

The first step of the present study was to evaluate the potential use of FA profiles of the adductor muscle (AM) of *C. edule* traded as fresh seafood for tracing their harvesting location. Results showed, for the first time, that it is possible to achieve the geographic origin with a spatial resolution < 10 Km without the complimentary use of stable isotope signatures. Besides, FA profile of the AM of fresh cockles showed to be able to discriminate the origin of specimens collected in close areas with different classifications according to European regulation (EC) No 1379/2013 for the capture/production of bivalves. This approach is paramount for traceability, expose fraud and ensure food safety. The way how the spatial distribution of *C. edule* among eight ecosystems along the Portuguese coast affects the FA profiles of the AM of this species and the temporal variability of FA profile between two consecutive years in areas within the same ecosystem were also tested. Data obtained from this research enable to differentiate cockles cultured in different Portuguese ecosystems, playing a key role for fishermen / producers willing to differentiate and add value to their products. Besides, this approach was able to discriminate the ecosystems which are microbiologically safer. The FA profiles presented inter-annual variability which must be considered for traceability as it compromise the discrimination of the geographic origin. In other attempt to ensure food safety, this study determined if the FA profile of the AM of live cockles displayed any significant shifts during the shelf-life (seven days post-harvest under a refrigerated environment) and how long post-harvest can these FA profiles be used to reliably trace their geographic origin. Results indicated that FA profiles remained stable until the third day post-harvest being able to be used to reliably trace geographic origin. After this period cockles started to exhibit contrasting FA profiles on their AM, namely a higher percentage of heptadecanoic acid (17:0), associated with the growth of pathogenic microorganisms responsible for food spoilage.

In this study, was also evaluated and validated the efficiency of TEF of shells from fresh bivalves as a proxy to discriminate the origin of specimens collected from adjacent areas of the same estuarine system. Barium (Ba), manganese (Mn), magnesium (Mg), strontium (Sr) and lead (Pb) were quantified in cockle shells. Results showed, for the first time, that this method can be used to achieve a reliable and accurate certification of origin for bivalves with a spatial resolution < 1 Km. TEF was also approached in the sense of evaluate if TEF of cockle shells from specimens captured in eight different ecosystems along the Portuguese Atlantic coastline can be used to successfully discriminate their geographic origin and if the temporal stability of TEF in cockle shells changes between two consecutive years in areas within the same ecosystem but displaying different classifications. TEF displayed by cockle shells successfully traced the geographic origin of cockles along the Portuguese coast and a periodical verification of TEF (> 6 months and < 1 year) is required to control temporal variability whenever comparing specimens originating from the same area collected more than six months apart.

The molecular tools developed during this study represent an economic benefit if and when applied to the bivalve production sector. The transfer of this technology to the bivalve's production constitutes a form of product's safety, promotion and differentiation, as well as a tool against fraud

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Abbreviations

Al	Aluminum
AL	Albufeira lagoon
ALA	Linolenic acid
AM	Adductor muscle
ANOSIM	One-way analysis of similarity
ANOVA	One-way analysis of variance
Ba	Barium
Ca	Calcium
CAP	Canonical analysis of principal coordinates
CH_2	Methylene group
CH_3	Methyl group
Co	Cobalt
COOH	Carboxyl group
Cr	Chromium
Cu	Copper
DB-1	Nonpolar DB-1ht columns
DB-FFAP	Nitroterephthalic-acid-modified polyethylene glycol (PEG)
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
E	Espinheiro channel
EC	European Council
EPA	Eicosapentaenoic acid
EU	European Union
FA	Fatty acid
FAME	Fatty acid methyl esters
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
H_2O_2	Hydrogen peroxide
Hg	Mercury
HNO_3	Nitric acid
HUFA	Highly unsaturated fatty acid
I	Ílhavo channel
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry

KOH	Potassium hydroxide
LA	Linoleic acid
LDA	Linear discriminant analysis
M	Mira channel
m/z	Mass-to-charge ratio
MANOVA	Multivariate analysis of variance
ME	Mira estuary
Mg	Magnesium
Mn	Manganese
MPN	Most probable number
MRC´s	Standards certified reference materials
MUFA	Monounsaturated fatty acids
NaCl	Sodium chloride
OL	Óbidos lagoon
p	p value
PA	Palmitic acid
Pb	Lead
PCO	Principal Coordinates Analysis
PCR-DGGE	Polymerase chain reaction denaturing gradient gel electrophoresis
PERMANOVA	Permutational Multivariate Analysis of Variance
PUFA	Polyunsaturated fatty acids
RAl	Ria de Alvor
RAv	Ria de Aveiro
RF	Ria Formosa
RSD	External precision estimates are based on %
SE	Sado estuary
SFA	Saturated fatty acids
SIMPER	Similarity Percentage analysis
SJ	São Jacinto channel
Sr	Strontium
TE	Tagus estuary
TEF	Trace element fingerprinting
U	Uranium
Zn	Zinc

CHAPTER 1. INTRODUCTION

Parts of the text of this chapter were published in the following publications:

Fernando Ricardo, Tânia Pimentel, Ana S. P. Moreira, Felisa Rey, Manuel A. Coimbra, M. Rosário Domingues, Pedro Domingues, Miguel Costa Leal & Ricardo Calado (2015). Potential use of fatty acids profiles of the adductor muscle of cockles (*Cerastoderma edule*) for traceability of collection site. *Scientific Reports 5*, 11125.

Fernando Ricardo, M. Rosário Domingues & Ricardo Calado (2017). Spatio-temporal variability in the fatty acid profile of the adductor muscle of the common cockle *Cerastoderma edule* and its relevance for tracing geographic origin. *Food Control 81*, 173-180.

Fernando Ricardo, Tânia Pimentel, Elisabete Maciel, Ana S.P. Moreira, M. Rosário Domingues & Ricardo Calado (2017). Fatty acid dynamics of the adductor muscle of live cockles (*Cerastoderma edule*) during their shelf-life and its relevance for traceability of geographic origin. *Food Control 77*, 192-198.

Fernando Ricardo, Luciana Génio, Miguel Costa Leal, Rui Albuquerque, Henrique Queiroga, Rui Rosa & Ricardo Calado (2015). Trace element fingerprinting of cockle (*Cerastoderma edule*) shells can reveal harvesting location in adjacent areas. *Scientific Reports 5*, 11932.

Fernando Ricardo, Tânia Pimentel, Luciana Génio & Ricardo Calado (2017). Spatio-temporal variability of trace elements fingerprints in cockle (*Cerastoderma edule*) shells and its relevance for tracing geographic origin. *Scientific Reports 7*, 3475.

1.1. Seafood trade

Fish and seafood products are important food resources (Usydus, Szlinder-Richert, Adamczyk, & Szatkowska, 2011) with relevant nutritional value and benefits for human health (Anacleto et al., 2014; Karl, Lehmann, Manthey-Karl, Meyer, & Ostermeyer, 2014; Özogul & Özogul, 2007). During the last decade, seafood production worldwide increased 25%, mainly due to the contribution of aquaculture production (FAO, 2016). From 2004 to 2013, the worldwide import of fishery products increased from 29.910 to 35.203 tons. The value of imports increased by 76%, from 71.4 million euros in 2004 to 126.0 million euros in 2013, while that of exports has grown from 29.8 to 36.4 tons, representing an increase by 22% in volume during 2004–2013 (Figure 1). The overall value of exports increased by 102% from 63.9 million euros (2004) to 129.3 million euros (2013).

Figure 1. Global import and export of fish and seafood products.

The continuous growth of the global seafood market promotes a number of challenges at an environmental, economic and societal level (Cao et al., 2015). As bivalve shellfish plays an important role in global fisheries and aquaculture (FAO, 2016), it is expected that supply chains commercializing live bivalves display a growing awareness towards food safety issues in an age of global trade (Oliveira, Cunha, Castilho, Romalde, & Pereira, 2011) (Figure 2).

Figure 2 illustrates the countries in which the network trade of bivalves (and derived products) was above 35.000 tons in the last four years, with real flow values being displayed between exporters and importers. China was the most representative country on the export of bivalves, with 500.000 tons, representing 1.804.388 million euros (ITC, 2014). Korea imported approximately 300.000 tons and France was the second largest importer with 273.000 tons. Bivalve products imported to Korea (from clams, cockles and scallops) were provided by China (Figure 2), reaching a value of 338.052 million euros. Mussels were the most important group of bivalves imported by France, mostly originating from Spain and the Netherlands, while scallops were provided from Asian countries, reaching values of 93.676 and 4.724 million euros respectively. The most exported bivalves to Portugal during 2010–2015 were clams and cockles. On the other side, the imports of bivalves to Portugal were mostly clams, cockles and mussels at 38.275.000 tons (representing 96% of the total imported bivalve's). The value of imported clams, cockles and mussels added up to 78 million euros, representing 88% of the total value of imported bivalve's by Portugal (Figure 2).

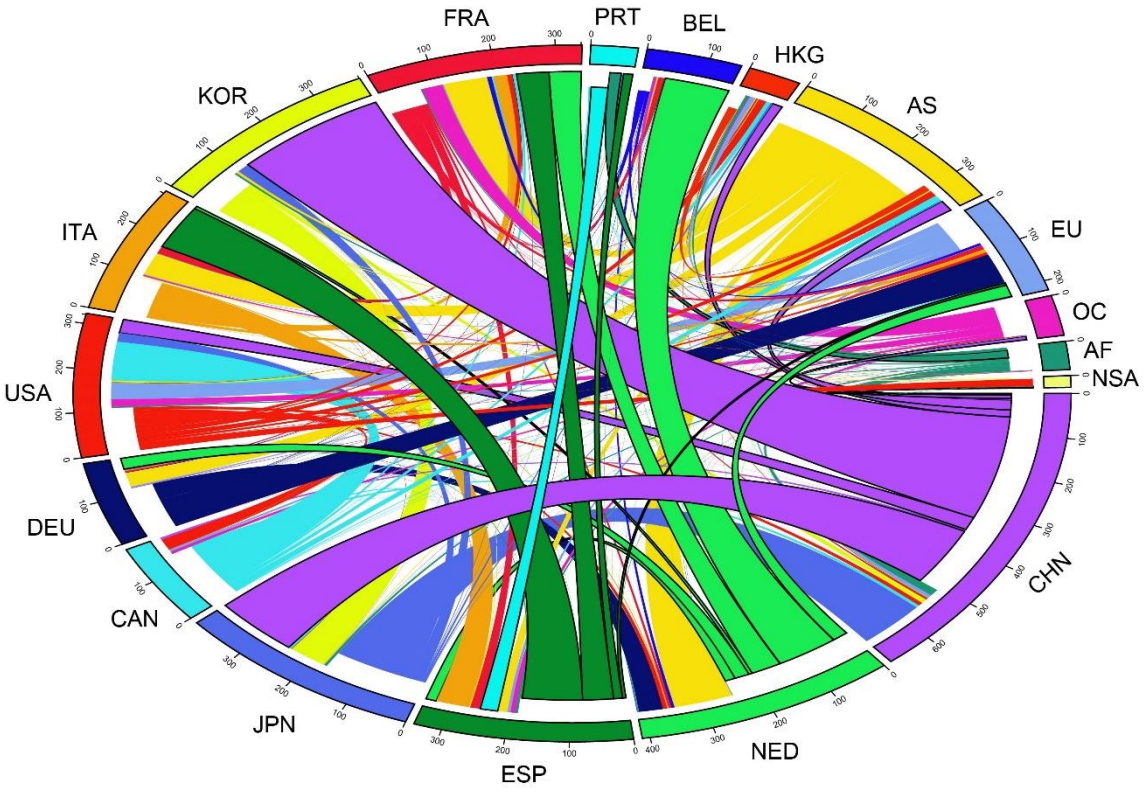

Figure 2. Chord diagram representing the global trade network of bivalves from 2012 to 2015 considering the twelve most relevant importers. China (CHN), Netherland (NED), Spain (ESP), Japan (JPN), Canada (CAN), Deutschland (DEU), United Stade of America (USA), Italy (ITA), Korea (KOR), France (FRA), Portugal (PRT), Belgium (BEL), Hong Kong (HKG), Asian (AS), Europe (EU), Oceania (OC), Africa (AF) and North Central and South America (NSA). Source (ITC, 2014).

The trading of bivalve's is associated with different levels of the supply chain. The primary stage contemplates concerns the producers and fishermen, while the second stage in the supply chain involves the depuration process, which is followed by a third and fourth stages corresponding to first and second buyers/processors and distributors, respectively, often followed by a distributor before bivalves reach the end consumer (Figure 3). The long paths from harvesting to consumption increase food safety risks inherent to bivalve products. Thus, in order to address current legislation and respond to the needs of the global market, it is important to develop efficient traceability tools associated with the fishing and aquaculture of bivalves, encompassing both processing and trading pathways.

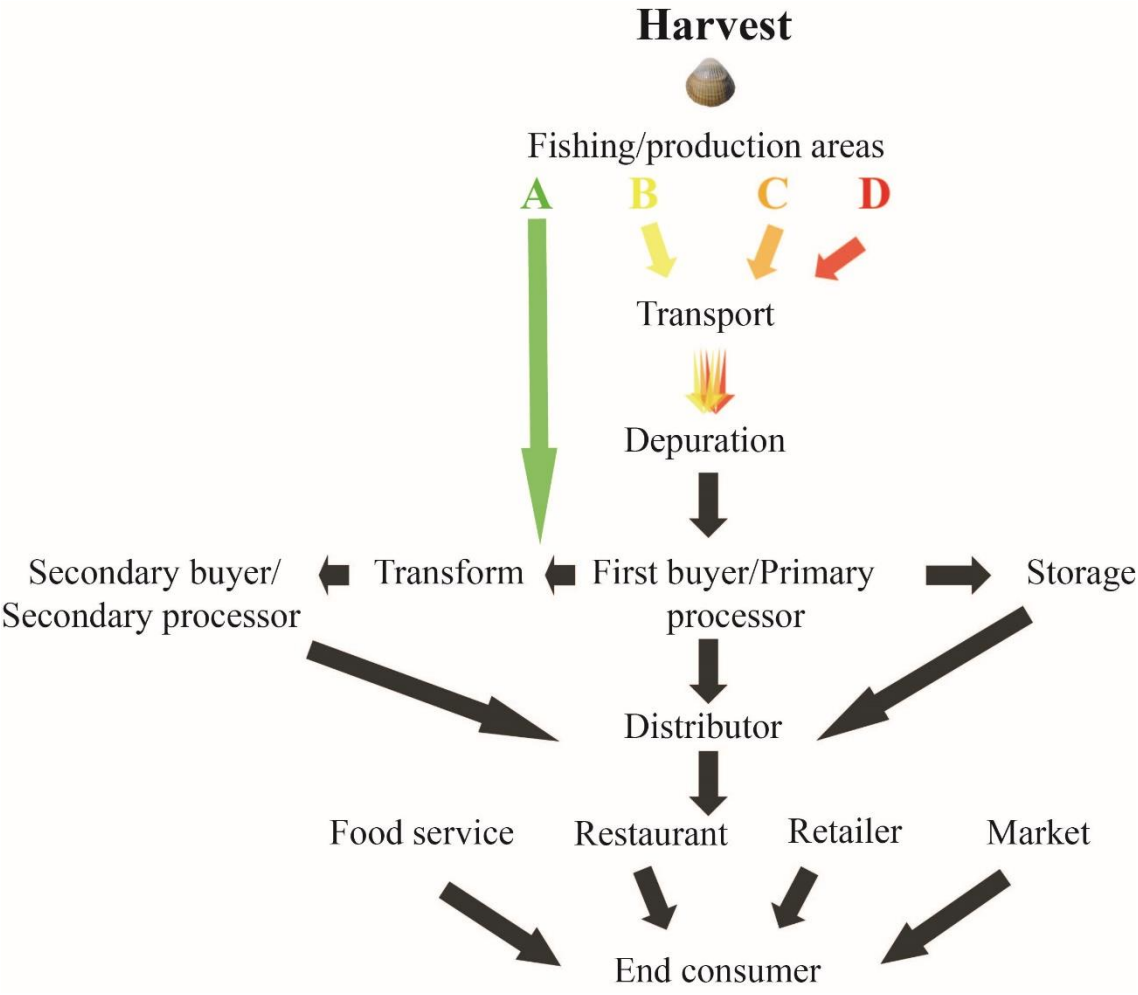

Figure 3. Bivalve supply chains and its many participants from fishermen/producers to the end consumer. Different colour of arrows, correspond to different fishing/production areas for bivalves in the EU and are ranked according to the levels of *Escherichia coli* present in the flesh and intra-valvular liquid of live specimens (for more details see section 1.2).

1.2. *Cerastoderma edule* as case study

The phylum Mollusca is one of the largest group in the animal kingdom with more than 50 000 described species (Gosling, 2004). The class Bivalvia is one of six classes of molluscs and contains about 7500 species (e.g. mussels, oysters, scallops, cockles and clams) (Gosling, 2004). By definition, bivalves possess two shells, called valves, joined together by a ligament along one edge, the hinge line that, in conjunction with interlocking "teeth" present in each valve, forms the hinge. Bivalves whose valves have the same shape (e.g. mussels, cockles and clams), valves are drawn together by an anterior and posterior adductor muscle (Tebble, 1966).

The mantle is responsible for secreting shell valves, ligament and hinge teeth. The shell is mainly formed by the deposition of calcium carbonate crystals in an organic matrix (conchiolin) and is structured in three layers: a thin outer periostracum of horny conchiolin, whose thickness is influenced by mechanical abrasion, fouling organisms, parasites or disease; a middle layer of aragonite or calcite (crystalline form of calcium carbonate); and an inner calcareous (nacreous) layer (Gosling, 2004; Tebble, 1966). Shell grows by the addition of material from the edge of the mantle and grows in thickness by deposition from all the mantle surface. Calcium is obtained from diet or taken up from water and carbonate from the CO_2/bicarbonate pool in the animal's tissues (Gosling, 2004). During growth their bivalves record information on environmental physico-chemical changes in their shells in the form of variable growth rates and geochemical properties (Chauvaud et al., 2005; Wanamaker, Kreutz, Schöne, & Introne, 2011; Williams, Arthur, Jones, et al., & Healy-Williams, 1982). Their shells serve not only for muscle attachment, but also for protection against predators and mechanical damage, as well as to keep mud and sand out of the mantle cavity in the case of burrowing species (Gosling, 2004).

In borrowing species, the foot has become modified for rapid and effective digging, and mantle edges are fused to form long siphons. Both these features allow the organisms to burrow deeply within different sediments while in species that live permanently attached to a substrate (e.g. oysters and mussels) the foot is very reduced (Gosling, 2004). Bivalves are mainly filter feeders, filtering actively water using two siphons (inhalant and exhalant) positioned on the surface of the sediment. Water is filtered through gills to keep phytoplankton, zooplankton and organic particles (Spencer, 2002).

The common cockle *Cerastoderma edule* (Linnaeus, 1758) is a species ecologically important species with high socio-economic relevance, as it supports a number of commercial fisheries. This ecological and economic relevance have been the main motivation for its selection as a model for the study. *C. edule* is an estuarine/marine species currently placed within family Cardiidae. Its two valves are very similar, solid, globular and broadly oval in outline, and may be up to 50 mm long. Shell presents 22-28 conspicuous broad ribs that are closely spaced. Outer surface off-white, yellowish or brownish (Tebble, 1966). Growth lines are prominent. Inner surface dull white, with a brownish or light purple stain on or about the posterior adductor muscle scar. The pallial line

lacks a sinus (Tebble, 1966). Both valves have two cardinal teeth, and shallow grooves on their inner surface run from their notched margin that, fade before reaching the pallial line (Tebble, 1966).

C. edule is one of the most common and widely distributed bivalve species in the estuaries and bays of the European Atlantic coastline (Malham, Hutchinson, & Longshaw, 2012; Mariani, Piccari, & De Matthaeis, 2002; Reise, 2003). Its preferential distribution goes from the middle to the lower intertidal region but, in some cases, it can still be found in subtidal areas. This species inhabits on clean sand, muddy sand, mud or muddy gravel sediments, burrowing to a depth of no more than 5 cm. It tolerates salinities ranging from 12.5 to 38.5 (Russell & Petersen, 1973) and temperatures between 4 and 38 ºC (Compton, Rijkenberg, Drent, & Piersma, 2007).

Cockles do not present external morphological differences between sexes, being dioecious and having a sex-ratio of approximately 1:1 in any given population (Boyden, 1971; Kandeel, Mohammed, Mostafa, & Abd-Alla, 2013). A large percentage of their population spawns at the same time, with adults typically beginning to spawn in their second summer; fertilisation is external, with oocytes and sperm being released into the water. Before undergoing metamorphosis into juvenile cockles and settle to the substrate, the free-swimming larvae (veliger larvae) lives for 3 to 5 weeks in the plankton (Creek, 1960). Growth rates vary with the season, being lower in winter, which leads to the marked growth-bands on the shell. Longevity for this species has been estimated to be of 6 years.

As already referred, the common cockle (*C. edule*) is commercially harvested in the soft-sediment shores of the European Atlantic coastline (Malham, Hutchinson, & Longshaw, 2012; Mariani, Piccari, & De Matthaeis, 2002; Reise, 2003), supporting several commercially important fisheries (Malham, Hutchinson, & Longshaw, 2012). Along the Portuguese coast (e.g. Ria de Aveiro, Óbidos lagoon, Ria de Alvor and Ria Formosa) the common cockle is the most commonly harvested bivalve and ranks among the most heavily collected molluscs for consumption. Its capture, has been increasing considerably in recent years (INE, 2014, 2015) and in 2015 5.000 tons were captured in national waters, corresponding to an estimated revenue of 4.5 million euros (INE, 2014, 2015).

1.3. Traceability associated with seafood safety risks

In an attempt to reduce supply chain risks and improve food safety and quality, the European Union (EU), one of the world´s territories with the highest seafood consumption per capita (FAO, 2016), established regulatory requirements launched in 2002 (EU directive 178/2002 EC (2002)). EU developed specific requirements for seafood traceability, particularly, article 58 of EC 1224/2009 which requires that "all lots of fisheries and aquaculture products shall be traceable at all stages of production, processing and distribution, from catching or harvesting to retail stage". More recently, the European regulation (EC) No 1379/2013 "on the common organization of the markets in fishery and aquaculture products" further contributed to the implementation of seafood traceability by requiring that the category of fishing gear or production method (i.e. caught or farmed) is provided together with geographic details of the catch area (EC, 2013). However, this information is not always available to end consumers and is prone to fraudulent use (e.g. mislabelling of place of origin). Consequently, even conscientious buyers aware of the potential hazards associated with the consumption of bivalves may not be able to securely purchase this highly-prized seafood. It is therefore critical to develop and validate reliable techniques that allow competent authorities to trace the origin of traded bivalves to ultimately fight fraud and prevent major risks to public health.

As already referred the global production of bivalves (e.g. cockles, clams, mussels and oysters) has notably increased since the 1990s, reaching over 16 million tons in 2014 (FAO, 2016). To protect public health and address current European legislation, it is paramount to trace the origin of captured/produced bivalves, not solely to a given estuary/coastal lagoon but specifically to its capture/production area. Bivalves are filter-feeders and are able to retain, accumulate and concentrate pathogens (e.g. *Salmonella and Vibrio*). The high trade volume of bivalves, combined with its raw or lightly cooked consumption, represents a potential risk to global human health (Rippey, 1994) and has been associated with outbreaks of a number of diseases, namely typhoid fever, hepatitis A, severe gastroenteritis and cholera (Iwamoto, Ayers, Mahon, & Swerdlow, 2010; Lees, 2000; Potasman, Paz, & Odeh, 2002).

The microbiological safety of bivalves destined for human consumption in member states of the European Union (EU) is covered by Council Regulation 853/2004 and 854/2004 (EC, 2004a, 2004b). Briefly, capture/production areas for bivalves in the EU are ranked according to the levels of *Escherichia coli* present in the flesh and intra-valvular liquid of live specimens and quantified through a 5-tubes 3-dilution most probable number (MPN) test. Bivalves originating from an area classified as "A" display less than 230 MPN of *E. coli* per 100 g of flesh and intra-valvular liquid. Consequently, these bivalves do not require any post-harvest treatment to reduce microbiological contamination. Bivalves originating from an area classified as "B" must not exceed in 90% of sampled specimens with 4600 MPN *E. coli* per 100 g of flesh and intra-valvular liquid, with the remaining 10% of specimens not exceeding 46000 MPN *E. coli* per 100 g of flesh

and intra-valvular liquid. As a result, these bivalves must be depurated, relayed or cooked by an approved method. Bivalves originating from an area classified as "C" must not exceed the limits of MPN test of 46.000 E. coli per 100 g of flesh and intra-valvular liquid. These bivalves must be relayed or cooked by an approved method (EC, 2004a, 2004b, 2005, 2008). In most EU countries, the fishing/production of bivalves is centered in estuaries and coastal lagoons, being common to have different classifications within the same aquatic system. Therefore, tracing the origin of traded bivalves to their specific fishing/production area, even within the same aquatic system, is paramount to ensure public food safety (Leal, Pimentel, Ricardo, Rosa, & Calado, 2015).

It is important to highlight that, during handling and storage, contamination of bivalves by enteric bacteria of human origin may also occur (Oliveira, Cunha, Castilho, Romalde, Pereira, 2011). In this way, these processes should be performed under controlled conditions to avoid contamination and growth of pathogenic microorganisms responsible for food spoilage (Emikpe, Adebisi, & Adedeji, 2011). Any change on the taste, smell, appearance or texture of bivalves can turn the product unacceptable and/or unsafe for the consumer (Costa, Conte, & Del Nobile, 2014). It is well known that the quality of live bivalves can be rapidly lost post-harvest, which ultimately conditions the shelf-life of these highly priced products (Lee, Lovatelli, & Ababouch, 2008). As live bivalves kept at ambient temperatures post-harvest often display a shorter shelf-life (< 48 h), they are most commonly stored on ice or refrigerated environments (0 to 4 ºC) to maintain their quality and safety for human consumption (Ashie, Smith, Simpson, & Haard, 1996; Rey, Miranda, Aubourg, & Barros-Velázquez, 2012). Under optimal storage conditions, live cockles maintain a fresh-like texture for approximately days post-harvesting (Ricardo et al., 2015a). Nevertheless, storage at lower temperatures does not impair biochemical reactions (e.g. enzymatic autolysis, lipid oxidation) or microbial growth that can affect the level of freshness of live bivalves (Ashie, Smith, Simpson, & Haard, 1996). About 4-5 days post-harvest live bivalves start to become slimy, produce an off odor, display an increase in pH, loose their water holding capacity and display a sharp decrease in their organoleptic acceptability (Ashie, Smith, Simpson, & Haard, 1996; Parveen et al., 2008).

The application of food traceability mechanisms, including biotechnological tools for authentication or origin of food products is becoming increasingly relevant, especially in seafood (Rasmussen & Morrissey, 2008; Teletchea, 2009). Notable biotechnological advances have been made but, despite the development of legal frameworks for seafood traceability, its implementation is still facing a number of challenges (Charlebois, Sterling, Haratifar, & Naing, 2014).

1.4. Biotechnological tools for seafood traceability

The global trade of seafood products, associated to a number of specific features of these highly priced goods, such as being highly perishable and being present in a large number of processed products, demanded the development of fast, simple and reliable analytical methodologies to trace them. Several different methods have been focused on biotechnological tools applied to seafood traceability, namely: biochemical (e.g. fatty acids; Olsen, Grahl-Nielsen, & Schander, 2009), geochemical (e.g. trace elemental fingerprinting; Sorte, Etter, Spackman, Boyle, & Hannigan, 2013), and DNA tools (e.g. PCR-DGGE; El Sheikha, Durand, Sarter, Okullo, & Montet, 2012), barcoding (Galimberti et al., 2013). The present work is focused solely on the use of biochemical and geochemical methods for seafood traceability.

Fatty acids (FA) are essential for life because they are important components of the plasma membrane, shifting its composition as a function of intrinsic (e.g. age, sex, reproductive cycle and phylogeny) and extrinsic (e.g. diet, temperature, depth and salinity) factors. Thus, as FA composition changes according to the physicochemical conditions of each particular environment, FA fingerprints can be useful for tracing geographic origin (Elsdon & Gillanders, 2003; Hall, Parrish, & Thompson, 2002). The diet available for aquatic organisms varies with habitat and ecosystem, affecting the FA composition of each organism (Bergé & Barnathan, 2005; Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003). Therefore it is paramount to use tissues that are less prone to be affected by seasonal variability (both environmental and dietary), such as the adductor muscle of bivalves (Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen, 2010; Olsen, Grahl-Nielsen, & Schander, 2009). The structural nature of the lipids presents in the AM of bivalves (mostly phospholipids and sterols) provides a more stable FA signature that is primarily determined by environmental conditions and functions of the cellular membrane, rather than dietary regimes (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003; Napolitano, Pollero, Gayoso, Macdonald, & Thompsan, 1997). At present, most studies available on the FA profiles of bivalves are focused on the analysis of the whole body or other organs than the AM (e.g. gonads, gills and digestive gland) (Napolitano, Macdonald, Thompson, & Ackman, 1992; Perez et al., 2013) that are regulated by intrinsic (e.g. age, phylogeny and sex) and external (e.g. diet, salinity and depth) factors (Olsen, Grahl-Nielsen, & Schander, 2009). Conversely, the FA signature of the AM is less prone to fast and dramatic shifts as compared to other organs (e.g. gonads and the digestive gland) (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003; Napolitano, Macdonald, Thompson, & Ackman, 1992). This biochemical approach is relatively low cost and fast once the FA extraction and quantification protocol has been optimized for the tissue of the target species (Ricardo et al., 2015a). However, lipids are susceptible to oxidation, which impair their use when monitoring processed products.

Trace element fingerprinting (TEF), uses the elemental profile recorded in hard biogenic structures, such as shells, statolith and otoliths (Carson et al., 2013; Reis-Santos et al., 2012).

Several trace elements are found in a wide range of marine species (Albuquerque, Queiroga, Swearer, Calado, & Leandro, 2016; Green et al., 2015; Norrie, Dunphy, Baker, & Lundquist, 2016; Ricardo et al., 2015b), with the most common ones being aluminium (Al), barium (Ba), calcium (Ca), cobalt (Co), chromium (Cr), copper (Cu), magnesium (Mg), manganese (Mn), lead (Pb), zinc (Zn) strontium (Sr) and uranium (U) (Génio, Simon, Kiel, & Cunha, 2015; Norrie, Dunphy, Baker, & Lundquist, 2016; Ricardo et al., 2015b). Considering that trace elements are influenced by the environmental features of each ecosystem (Takesue, Bacon, & Thompson, 2008) and that these mineral structures grow throughout the year, TEF represents a low cost, fast, reliable and accurate method, that has already been successfully used to distinguish specimens geographically close from populations (Becker, Fodrie, McMillan, & Levin, 2004; Sorte, Etter, Spackman, Boyle, & Hannigan, 2013; Zacherl, 2005). However, the need of hard structures to successfully use geochemical tools limits their applicability to processed products (e.g. canned food and fish fillets).

1.4.1. Fatty acids fingerprints as natural tags

Lipids are one of the largest groups of nutrients found in seafood products and play several important biological functions, namely storage and transport of energy, formation of cell membranes, maintenance of their structural integrity, synthesis of prostaglandins and transport of fat-soluble vitamins (Arts, Brett, & Kainz, 2009; Nunes, Bandarra, & Batista, 2011). Considering their functions in living organisms they can be divided in two main groups: nonpolar lipids (e.g. acylglycerols, free (nonesterified) fatty acids, sterols, wax and steryl esters) and polar lipids (glycolipids and phospholipids) (Arts, Brett, & Kainz, 2009). Polar lipids are important structural components of cell membranes acting as a selective permeable barrier for cells and organelles. These lipids have an important role in specific membrane functions providing the matrix for a very wide variety of metabolic processes, being directly involved in membrane fusion events. Besides, some polar lipids participate in cell signalling pathways (e.g. inositol lipids, sphingolipids, oxidative products) acting as key intermediates or precursors of intermediates and play a role in responding to changes in the environment (Arts, Brett, & Kainz, 2009). Nonpolar lipids can be easily catabolised to provide metabolic energy, being triacylglycerol's abundant storage products (Lucy, 1974).

Fatty acids (FA) are the main constituents of polar lipids. Its composition is determinant for the physical properties, stability and nutritional value of the lipid fraction present in any food itens. FA are carboxylic acids (carboxyl group -COOH) with a more or less long chain of carbons, (4 to 36 carbons) attached to hydrogen (methylene group CH_2) and ending with a methyl group (CH_3) (Koolman, Röhm, Wirth, & Robertson, 2005) (Figure 4). These compounds can be classified as saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA), depending on the number of double bonds in the carbon skeleton, zero, one or more than one, respectively.

SFA are characterized by the absence of double bonds in the carbon chain, which gives rise to a flat molecular structure. Among unsaturated fatty acids, MUFA differ from PUFA because they have only one double bond in the carbon chain whereas PUFA have 2 or more double bonds. The letter *n* (*n*-) is used to indicate the distance, in carbon atoms, from the double bond to the terminal methyl group, identifying the different FA families (Figure 4).

FA can also be classified as non-essential (easily catabolised to provide metabolic energy) and essential, when they are not synthesize by the organism and are exclusively acquired from ingested food (Arts, Brett, & Kainz, 2009). PUFA, such as linoleic acid (18:2*n*-6) and linolenic acid (18:3*n*-3) as well as eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), are essential FA that are vital for maintaining somatic and population growth, survival, and reproductive success (Arts, Brett, & Kainz, 2009). Fish and shellfish are dominated by PUFA, followed by SFA and MUFA, whose proportions and amounts are regulated by intrinsic (e.g. age, phylogeny and sex) and external (e.g. diet, salinity and depth) factors (Olsen, Grahl-Nielsen, & Schander, 2009). In marine organisms, such as bivalves, palmitic acid (16:0; Figure 4a) is the most relevant within the SFA group, while oleic acid (18:1*n*-9; Figure 4b) is the dominant in MUFA, and eicosapentaenoic acid (EPA, 20:5*n*-3; Figure 4d) and docosahexaenoic acid (DHA, 22:6*n*-3; Figure 4e) are the most well represented within PUFA (Galap, NetchitaïLo, Leboulenger, & Grillot, 1999; Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen, 2010; Nunes, Bandarra, & Batista, 2011; Olsen, Grahl-Nielsen, & Schander, 2009).

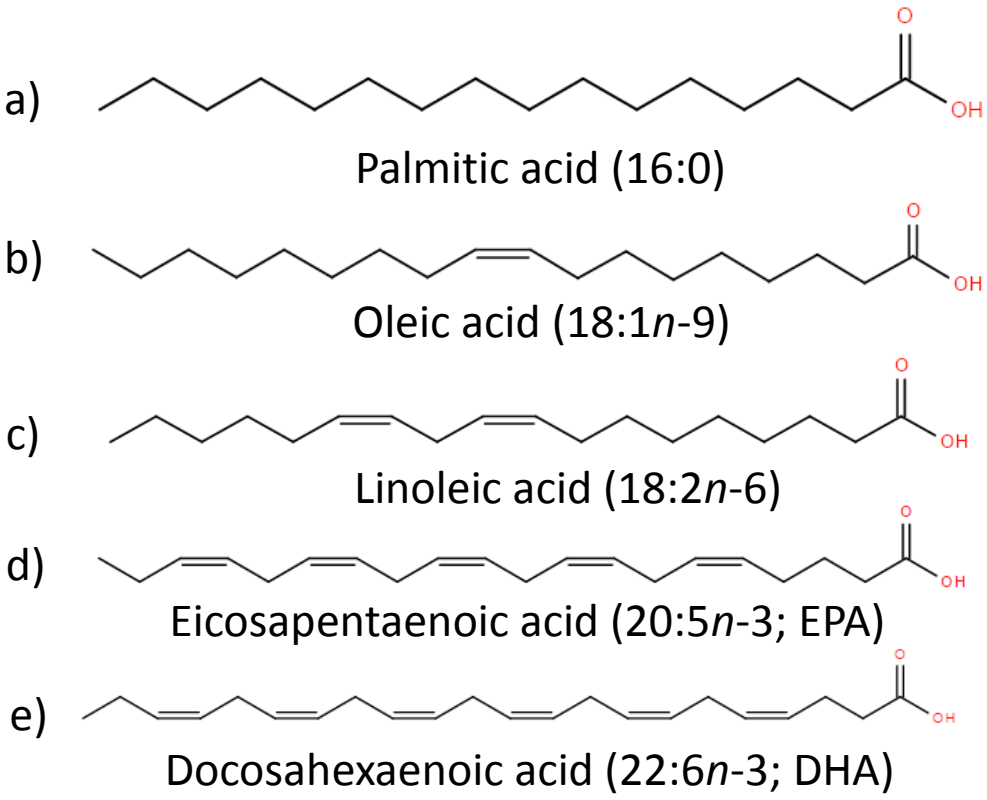

Figure 4. Molecular structures: a) saturated fatty acids (SFA), b) monounsaturated fatty acids (MUFA) and c,d,e) polyunsaturated fatty acids (PUFA).

Bivalves are not able to synthesize a number of *n*-3 and *n*-6 PUFA that are vital, for their growth, reproduction and immunity. Thus, FA composition in bivalves is generally related with the availability and composition of their natural diet, water temperature, water depth, salinity and reproductive cycle (Caers, Coutteau, & Sorgeloos, 2000; Prato, Danieli, Maffia, & Biandolino, 2010). Algae (e.g. phytoplankton) are primary producers at the base of the food chain and being paramount for primary consumers due to their ability to biosynthesize several PUFA *de novo* (Parrish, 2013). Oleic acid (18:1*n*-9) is the precursor of all *n*-3 and *n*-6, which via Δ9 and Δ12 desaturases to produce 18:2*n*-6 (linoleic acid), which can then be further desaturated by Δ15 desaturase to give origin to 18:3*n*-3 (α-linolenic acid) (Bergé & Barnathan, 2005). Desaturations at the Δ6 and Δ5 positions in the carbon backbone, and an intermediate two carbon chain elongation are the pathways from 18:2*n*-6 to arachidonic acid and from 18:3*n*-3 to eicosapentaenoic acid (EPA, 20:5*n*-3), while the production of docosahexaenoic acid (DHA, 22:6*n*-3) from eicosapentaenoic acid requires an additional desaturation (Δ4) and two carbon chain elongation (Adarme-Vega, Thomas-Hall, Lim, & Schenk, 2014; Graham, Cirpus, Rein, & Napier, 2004). In the alternative pathway, the elongation (Δ9) step precedes desaturation (Δ9), with linoleic acid and α-linolenic acid being elongated to C20 forms which then undergo two sequential desaturations (Δ8 and Δ5-desaturation) (Figure 5).

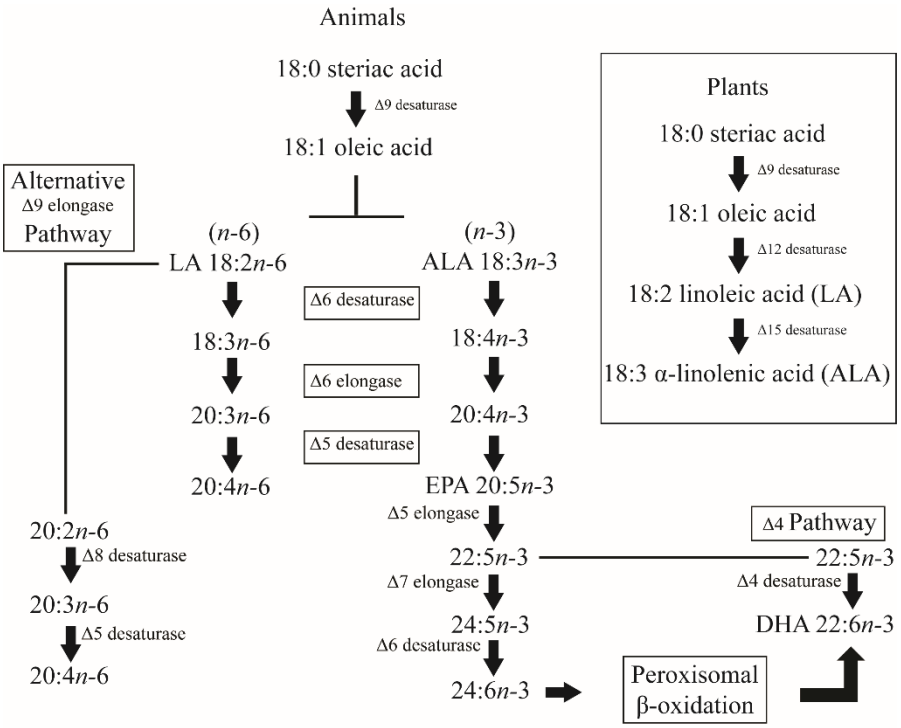

Figure 5. Biosynthesis of long chain *n*-3 polyunsaturated fatty acids (PUFA). The precursor's linoleic acid (LA) and α-linolenic acid (ALA) are the predominant fatty acids synthesised by algae. These then enter the bivalve food web and are subsequently metabolised to C20+ PUFA (Adarme-Vega, Thomas-Hall, Lim, & Schenk, 2014; Graham, Cirpus, Rein, & Napier, 2004).

FA profile of bivalves can provide information about the nutritional value of the food source as well its geographic origin. The structural nature of the lipids present in the AM of bivalves (mostly phospholipids and sterols) provides a stable FA signature that is mainly determined by environmental conditions and functions of the cellular membrane than by dietary regimes (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003; Napolitano, Pollero, Gayoso, Macdonald, & Thompson, 1997). Indeed, salinity and temperature are known to the structure and fluidity of cell membranes, with higher saline fluctuations and/or lower water temperatures promoting a decrease in the levels of saturated FA (SFA), which are responsible to stabilize the bilayer structure, and an increase in the concentration of polyunsaturated FA (PUFA), which enhance the bilayer fluidity (Nemova, Fokina, Nefedova, Ruokolainen, & Bakhmet, 2013). For this reason, the FA signature of the AM is less prone to fast and dramatic shifts comparatively with other organs in bivalves.

1.4.2. Trace element fingerprints as natural tags

Information on environmental conditions can be retrieved through geochemical signals recorded in hard mineral structures, such as coral skeletons (Gaetani & Cohen, 2006; Swart & Grottoli, 2003; Swart et al., 1999; Thresher, Fallon, & Townsend, 2016), fish otoliths (Campana & Thorrold, 2001; Peacock et al., 2016; Reis-Santos et al., 2012; Riou et al., 2016), foraminifera test (Lea, Shen, & Boyle, 1989; Ni Fhlaithearta, Ernst, Nierop, Lange, & Reichart, 2013) and bivalves shells (Füllenbach, Schöne, & Mertz-Kraus, 2015; Lazareth, Le Cornec, Candaudap, & Freydier, 2013; Lazareth, Putten, André, & Dehairs, 2003; Putten, Dehairs, Keppens, & Baeyens, 2000), as they accrete through time under the influence of their surrounding environmental conditions.

Indeed, bivalves record and reflect a large amount of environmental information in their shells and, for this reason, are used as climate and environmental proxy archives (Schöne & Gillikin, 2013; Wanamaker, Kreutz, Schöne, & Introne, 2011). During growth bivalves, deposit new layers of shell that mirror the chemical composition of their environmental conditions at the time they formed. Biomineralization in bivalve's takes place in a thin film of liquid, the extra pallial fluid, located between the calcifying shell surface and the mantle epithelium (Figure 6) (Wheeler, 1992). The inner and outer and/or middle shell layers are precipitated from the inner and outer extra pallial fluid, respectively. Extra pallial fluid is isolated from seawater and, for this reason, both present contrasting elemental concentrations (Wilbur & Saleuddin, 1983). Chemical elements present in seawater enter the haemolymph primarily through the bivalve gills; these elements later move into the extra pallial fluid through the epithelial mantle cells (Wilbur & Saleuddin, 1983). Chemical elements in seawater may also enter the bivalve through their gut or be directly uptaken by the mantle outer epithelium (Wilbur & Saleuddin, 1983).

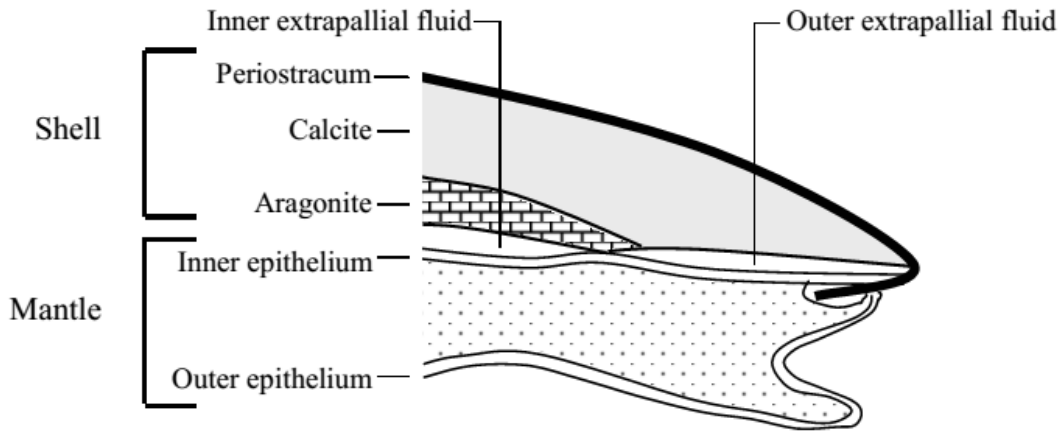

Figure 6. Illustration of a cross-section through a bivalve shell with the different shell layers (aragonite and calcite), the mantle, and the sites of calcification (central or inner extra pallial fluid and marginal or outer shown (figure from Gillikin, 2005).

The growth rate of bivalves is influenced by several variables, such as temperature and food supply/quality (e.g. Butler et al., 2010; Mette, Wanamaker, Carroll, Ambrose, & Retelle, 2016; Schöne et al., 2004; Witbaard, Duineveld, & De Wilde, 1999; Witbaard, Franken, & Visser, 1997); moreover, there is a positive correlations between shell growth and higher water temperatures and food availability (i.e. Marali & Schöne, 2015; Schöne et al., 2005; Witbaard, Franken, & Visser, 1997). Bivalves deposit their biogenic carbonates on a periodic basis and at different rates (Deith, 1985; Thompson, Jones, & Dreibelbis, 1980), with periods of fast growth contrasting with those of a slower growth (Schöne, 2008; Schöne & Gillikin, 2013). The periodicity of deposition displayed by such structures can ranges from daily to annual (Campana & Thorrold, 2001; Gordon & Carriker, 1978).

Trace element fingerprints (TEF) of biogenic carbonates have been successfully used as "natural tags" to discriminate specimens from different geographical origins (Ricardo et al., 2015b; Zacherl, 2005). TEF variability in hard mineral structures such as shells of marine bivalves is influenced by the availability of trace elements in seawater, which, as already referred, reflect shifts in the environmental features of their ecosystems (Lloyd et al., 2008; Zacherl, Morgan, Swearer, & Warner, 2009). Being inert structures, TEF displayed by bivalve shells are chronological fingerprints that reflect the geographical surroundings of a given specimen from birth to its harvest (Becker, Fodrie, McMillan, & Levin, 2004; Génio, Simon, Kiel, & Cunha, 2015; Strasser, Mullineaux, & Thorrold, 2008; Zacherl, 2005). A wide range of element/calcium ratios are commonly recorded in these calcified structures, the most common ones being Ba/Ca, Cd/Ca, Cu/Ca, Cr/Ca, Mg/Ca, Mn/Ca, Pb/Ca, Sr/Ca, U/Ca and Zn/Ca (Carson, 2010; Génio, Simon, Kiel,& Cunha, 2015; Ricardo et al., 2015b). Among these ratios the Mg/Ca and Sr/Ca are likely the most well studied, being proposed as salinity independent temperature proxies in biogenic carbonates. The Ba/Ca and Mn/Ca ratios are often associated with phytoplankton variability, being the last one, as well as the ratio Pb/Ca, associated with anthropogenic pressures

(historical metal industries and acute pollution from boats using leaded gasoline, respectively) (Bourgoin, 1990; Pitts & Wallace, 1994; Vale, Canário, Caetano, Lavrado, & Brito, 2008). The presence of other metals in bivalve shells, such as Cu, Zn and Cd, have also been shown to be promising proxies for the flagging of environmental pollution (Richardson, Chenery, & Cook, 2001).

1.5. Objectives

Market globalization and recurrent alerts on food safety issues resulted in a growing awareness of consumers on the need for food traceability. This is particularly relevant for seafood due to its highly perishable nature and importance as the main protein supplier of world's population in 21st century. In 2013, the global production of bivalves from fisheries and aquaculture reached approximately 14 million tonnes worldwide with a monetary value of over 55 million euros. In Portugal, around 7.500.000 tons were captured valued in 9.2 million euros in 2013. The present study aims to develop efficient protocols for the traceability of bivalves either produced or harvested in ecosystems along Portuguese coast (see Appendix A for a detailed descriptions of each study area) according to the following null hypotheses:

I. The fatty acid profile of the adductor muscle of *C. edule* does not differ between specimens captured in different channels of Ria de Aveiro (section 2.1);
II. The fatty acid profile of the adductor muscle of *C. edule* does not present spatio-temporal variability (section 2.2);
III. The fatty acid profile of the adductor muscle of *C. edule* does not changed during the shelf-life (section 2.3);
IV. The trace element fingerprints of *C. edule* shells does not differ between specimens captured in different locations of Ria de Aveiro (section 3.1);
V. The trace element fingerprints of *C. edule* shells does not present spatio-temporal variability (section 3.2);

CHAPTER 2. THE USE OF FATTY ACID PROFILES IN BIVALVES TRACEABILITY

2.1. POTENTIAL USE OF FATTY ACID PROFILES OF THE ADDUCTOR MUSCLE OF COCKLES (*CERASTODERMA EDULE*) FOR TRACEABILITY OF COLLECTION SITE

2.2. SPATIO-TEMPORAL VARIABILITY IN THE FATTY ACID PROFILE OF THE ADDUCTOR MUSCLE OF THE COMMON COCKLE *CERASTODERMA EDULE* AND ITS RELEVANCE FOR TRACING GEOGRAPHIC ORIGIN

2.3. FATTY ACID DYNAMICS OF THE ADDUCTOR MUSCLE OF LIVE COCKLES (*CERASTODERMA EDULE*) DURING THEIR SHELF-LIFE AND ITS RELEVANCE FOR TRACEABILITY OF GEOGRAPHIC ORIGIN

CHAPTER 2. THE USE OF FATTY ACID PROFILES IN BIVALVES TRACEABILITY

2.1. Potential use of fatty acid profiles of the adductor muscle of cockles (*Cerastoderma edule*) for traceability of collection site

The material & methods, results and discussion presented in this section were integrally published as follow:

Fernando Ricardo, Tânia Pimentel, Ana S. P. Moreira, Felisa Rey, Manuel A. Coimbra, M. Rosário Domingues, Pedro Domingues, Miguel Costa Leal & Ricardo Calado (2015). Potential use of fatty acids profiles of the adductor muscle of cockles (*Cerastoderma edule*) for traceability of collection site. *Scientific Reports 5*, 11125.

2.1.1. Background and aim of the study

Market globalization and recurrent alerts on food safety issues resulted in a growing awareness of consumers on the need for food traceability. This is particularly relevant for seafood due to its highly perishable nature and importance as the main protein supplier of world's population in 21st century. Ria de Aveiro is a coastal lagoon located in the western Atlantic margin of Portugal (Figure 7) where bivalve fisheries/aquaculture play an important socio-economic role (Pereira, Maia, & Gaspar, 2013), especially the harvesting of cockle (*Cerastoderma edule*) from Ria de Aveiro, which exceeds 1000 tons per year. This coastal lagoon currently has four official bivalve capture/production areas classified either as B or C (see definition above). To provide a potential tool for bivalve's traceability, the present study aimed to evaluate if the FA profile of the AM of fresh cockles could be used as a method to discriminate the origin of specimens collected in different channels of Ria de Aveiro, either with identical or different classifications for bivalve capture/production. The following null hypothesis was tested: the FA profile of the AM of *C. edule* does not differ between specimens captured in different channels of Ria de Aveiro.

2.1.2. Material and methods

2.1.2.1. Study area and sample collection

Samples of *C. edule* were collected in important fishing areas during June 2013 within four main channels (São Jacinto, Mira, Ilhavo and Espinheiro; Figure 7) of Ria de Aveiro (Northwestern coast of Portugal), with their current classification under the legislation for shellfish production waters being used as rationale for the experimental design used (see Introduction). During the study period, the channels of São Jacinto and Mira were classified by Portuguese authorities as "B", while those of Ilhavo and Espinheiro were classified as "C" (DL, 2013). Two areas were surveyed in each channel (Figure 7; Table S1 on appendix B), and four cockles collected per area (4 channels X 2 areas X 4 replicates = 32 samples). All samples were collected by hand-raking and stored in aseptic food grade plastic bags that were kept refrigerated during sampling and transportation for processing in the laboratory on the same day. Bivalves were dissected to extract the AM, which was then stored at − 80 °C for subsequent FA analysis.

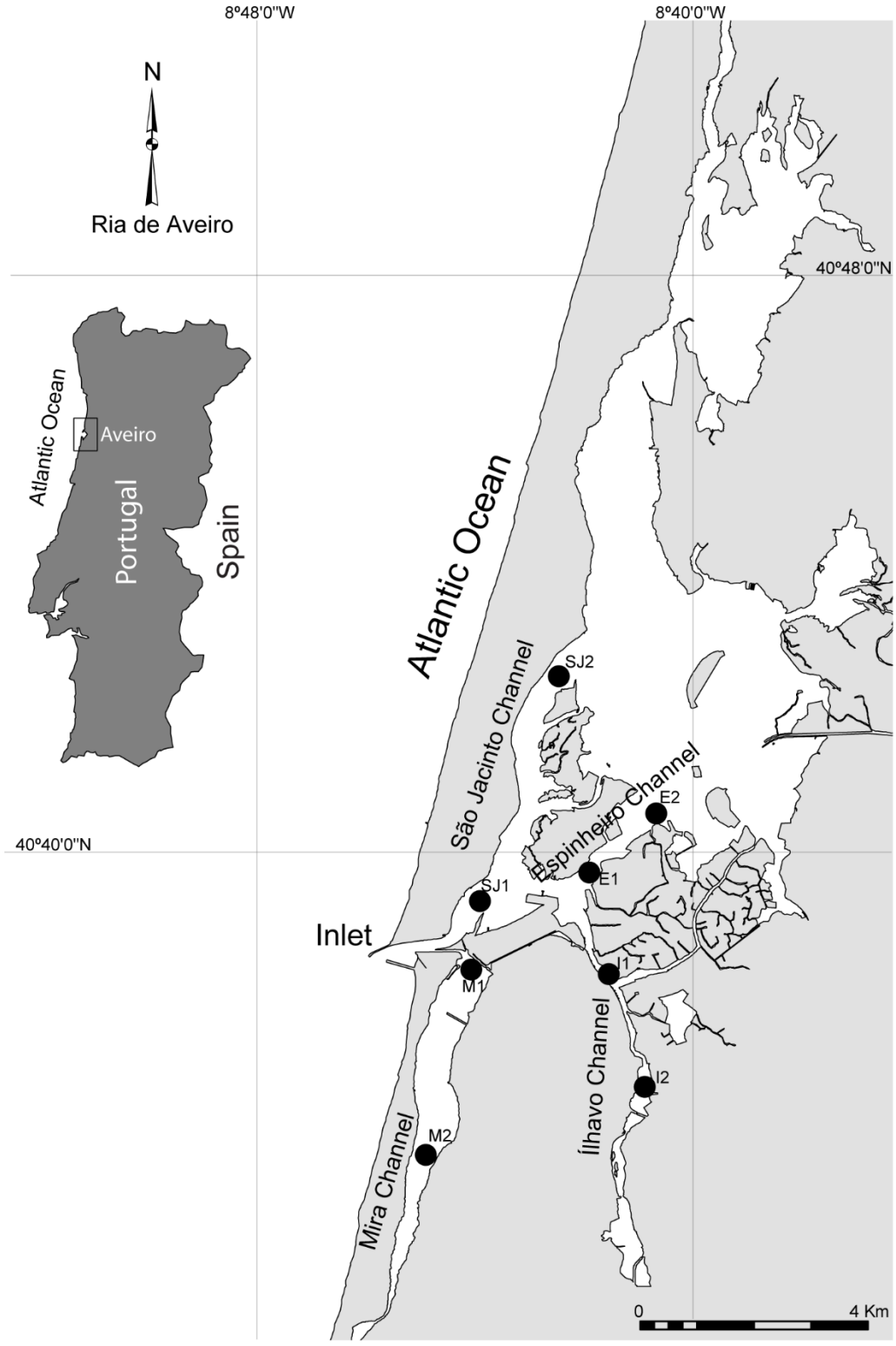

Figure 7. Sampling locations of *Cerastoderma edule* in Ria de Aveiro, Portugal: São Jacinto (SJ1: 40°39′ 23.70″ N, 8°43′ 49.40″ W and SJ2: 40°42′ 34.00″ N, 8°42′ 24.10″ W), Mira (M1: 40°38′ 26.30″ N, 8°43′ 58.90″ W and M2: 40°35′ 58.30″ N, 8°44′ 47.80″ W), Ilhavo (I1: 40°38′ 22.36″ N, 8°41′ 24.93″ W and I2: 40°37′ 03.10″ N, 8°40′ 48.00″ W) and Espinheiro (E1: 40°39′ 48.50″ N, 8°41′ 45.03″ W and E2: 40°40′ 37.10″ N, 8°40′ 28.90″ W). The map was created using the software ArcGIS v9.2.

2.1.2.2. Fatty acids analysis

All samples were freeze dried prior to biochemical analysis. Total lipids of the AM of each individual were extracted in methanol/chloroform (2:1 v/v) following Bligh & Dyer (1959). Fatty acids methyl esters (FAMEs) of the total lipid extracts were obtained by transmethylation according to the method described by Aued-Pimentel, Lago, Chaves, & Kumagai (2004). Briefly, 15 µ g of dried lipid extract was dissolved in 1 mL n-hexane, 0.2 mL of methanolic solution KOH (2 M) and 2 mL satured NaCl solution, followed by intense vortexing. After centrifugation at 2000 rpm for 5 min, the organic phase was collected and dried under a nitrogen stream. The resulting FAMEs were dissolved in hexane prior to injection and analysed by gas chromatography-mass spectrometry (GC-MS) on an Agilent Technologies 6890N Network (Santa Clara. CA) equipped with a DB-1 column with 30 m length, 0.25 mm internal diameter and 0.1 µm film thickness (J&W Scientific, Folsom, CA). The GC-MS was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 40-500 in a 1 s cycle in a full scan mode acquisition. The column temperature was programmed from 40 °C initial oven temperature at 20 °C min^{-1} to 220 °C, then from 220 to 240 °C at 2 °C min^{-1} and then from 240 to 260 °C at 5 °C min-1. The detector was set at 230 °C and the injector at 220 °C. Helium was used as carrier gas at a flow rate of 1.7 mL min^{-1}. Individual FA peaks were integrated using the equipment's software, and identified considering the retention time and mass spectrum of each FA relative to 34 mixed FA standards (C6-C24, Supelco 37 Component Fame Mix). The areas of the 20 selected FAMEs were integrated setting response factor to 1. Values of FA were reported as mean values ± standard deviation (SD) and expressed as relative percentages of the total pool of fatty acids.

2.1.2.3. Statistical analysis

Biochemical data were represented by the relative abundance of FA per replicate, per area for each channel. The resemblance matrix among samples was obtained with the Bray-Curtis similarity coefficient, following a log (x + 1) transformation in order to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson, 2008). A preliminary one-way analysis of similarity (ANOSIM) was performed to detect significant differences in FA profiles of *C. edule* between sampling areas within the same channel. Briefly, ANOSIM calculates a global R statistic that assesses the differences between groups, where values close to one indicate maximum differences between groups and values near zero suggest complete groups overlap (Clarke & Gorley, 2006). As no significant differences were recorded between areas within the same channel, all samples per channel were pooled, i.e. a total of 8 replicates per channel (see Table S2 on appendix B).

The differences in FA profile in *C. edule* between channels were analysed by ordination analysis, using Principal Coordinates Analysis (PCO). This analysis allows the visualization of inter-individual differences in FA profiles, representing differences between all channels from each FA along the first two axes. ANOSIM was also used (see above for details) to detect differences in FA profiles of the AM of *C. edule* among channels. One-way analysis of variance (ANOVA) was used to assess differences among channels for each individual FA after confirming normality with the Shapiro test and homogeneity of variance with the Bartlett test. Post hoc Bonferroni test was used when ANOVA revealed significant differences ($p < 0.05$).

Similarity percentages (SIMPER) were determined to describe the differences in individual and classes of FA among channels. SIMPER identifies the FA that contribute most to the variations in the assemblage patterns recorded. Only the FA that cumulatively contributed up to 80% of the dissimilarities recorded were selected to characterize the differences in the FA profile of cockles from different channels (Clarke & Gorley, 2006). ANOVAs were performed using GraphPad Prism 6 (GraphPad Software. Inc. San Diego, CA, USA), while all multivariate statistical tests (ANOSIM, PCO and SIMPER) were performed using PRIMER v6 with the add-on PERMANOVA+

2.1.3. Results

Saturated FA (SFA) represented 28–41% of all FA identified in the AM of *C. edule* from different channels, whereas mono-unsaturated FA (MUFA) represented 12–13% (Table 1). Polyunsaturated FA (PUFA) were the most abundant class of FA recorded in the bivalves surveyed as their levels ranged between 45 and 58% of the total pool of FA. The major SFA were palmitic (16:0; PA) and stearic acid (18:0), which represented over 50% of all SFA recorded in the AM of cockles and varied significantly ($p < 0.05$) among channels (Table 1). The dominant MUFA were elaidic (18:1*n*-9 trans) and eicosenoic acid (20:1*n*-9), and their content in the AM of cockles was similar across channels (Table 1). The most abundant PUFA were eicosapentaenoic (EPA) (20:5*n*-3) and docosahexaenoic acid (DHA) (22:6*n*-3), which together accounted for over 60% of total PUFA and, at least, 35% of the total pool of FA.

Table 1. Fatty acid profile (data presented as percentage of relative abundances) of the adductor muscle of *Cerastoderma edule* (values are means of 8 replicates ± SD) from São Jacinto (SJ), Mira (M), Ilhavo (I) and Espinheiro (E) channels in Ria de Aveiro, Portugal. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; and PUFA – polyunsaturated fatty acids. Values of p highlighted in light grey are < 0.05.

Fatty Acid	**São Jacinto**	**Mira**	**Ilhavo**	**Espinheiro**	**Bonferroni' s pairwise comparisons**					
(%)	**(SJ)**	**(M)**	**(I)**	**(E)**	**SJ vs M**	**SJ vs I**	**SJ vs E**	**M vs I**	**M vs E**	**I vs E**
14:0	2.61 ± 1.30	1.75 ± 0.89	1.57 ± 0.99	2.40 ± 1.23	0.0532	0.0701	>0.9999	>0.9999	0.2342	0.2889
15:0	0.72 ± 1.07	0.30 ± 0.13	0.42 ± 0.27	0.34 ± 0.12	0.4322	>0.9999	0.7714	>0.9999	>0.9999	>0.9999
16:0	24.38 ± 5.92	17.76 ± 2.96	14.51 ± 4.10	14.51 ± 6.85	0.0257	<0.0001	<0.0001	0.4131	0.0237	>0.9999
17:0	1.05 ± 0.24	0.90 ± 0.27	1.50 ± 0.44	1.25 ± 0.42	>0.9999	0.4840	>0.9999	0.0542	0.5449	>0.9999
18:0	12.66 ± 1.64	12.87 ± 1.24	11.54 ± 1.71	9.93 ± 2.87	>0.9999	>0.9999	0.0632	0.8207	0.0284	>0.9999
∑SFA	**41.42 ± 6.15**	**33.58 ± 4.17**	**29.54 ± 6.71**	**28.43 ± 10.81**	0.1747	0.0021	<0.0001	0.7540	0.0745	>0.9999
16:1*n*-9	3.10 ± 1.20	2.81 ± 0.88	2.95 ± 0.96	3.03 ± 1.54	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
18:1*n*-9c	0.69 ± 0.25	0.76 ± 0.23	0.71 ± 0.23	0.54 ± 0.16	>0.9999	0.3857	>0.9999	0.8176	>0.9999	0.0283
18:1*n*-9t	3.08 ± 0.90	3.16 ± 0.34	2.90 ± 0.66	3.01 ± 0.57	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
20:1*n*-9	5.38 ± 1.27	6.56 ± 1.09	6.39 ± 0.57	5.97 ± 0.67	0.4559	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
∑MUFA	**12.25 ± 1.85**	**13.29 ± 1.23**	**12.95 ± 1.19**	**12.55 ± 1.99**	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
18:3*n*-6	0.50 ± 0.32	0.58 ± 0.27	0.65 ± 0.33	0.65 ± 0.35	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
18:2*n*-6	0.25 ± 0.12	0.26 ± 0.07	0.33 ± 0.15	0.30 ± 0.07	>0.9999	0.4382	>0.9999	0.4890	>0.9999	0.8026
20:5*n*-3	22.90 ± 1.80	21.16 ± 2.19	22.52 ± 1.56	24.07 ± 3.66	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
20:3*n*-3	0.75 ± 0.16	0.94 ± 0.23	1.05 ± 0.27	0.80 ± 0.14	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
20:2*n*-9	0.53 ± 0.23	0.59 ± 0.19	0.54 ± 0.13	0.59 ± 0.13	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
21:5*n*-3	0.95 ± 0.27	0.92 ± 0.26	1.02 ± 0.33	0.92 ± 0.30	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
22:6*n*-3	12.98 ± 3.63	15.97 ± 3.40	18.70 ± 4.04	22.07 ± 8.52	0.2539	<0.0001	<0.0001	0.0819	0.0531	>0.9999
22:4*n*-6	0.22 ± 0.11	0.25 ± 0.09	0.78 ± 0.93	0.35 ± 0.14	>0.9999	0.9282	>0.9999	>0.9999	>0.9999	>0.9999
22:4*n*-3	0.97 ± 0.37	1.71 ± 0.46	2.11 ± 0.73	1.94 ± 0.99	0.0107	0.0001	0.0023	>0.9999	>0.9999	>0.9999
22:3*n*-6	0.74 ± 0.27	1.30 ± 0.32	2.11 ± 1.41	1.42 ± 0.59	0.0341	0.0003	0.0113	0.8560	>0.9999	>0.9999
22:2*n*-9	4.21 ± 1.81	4.75 ± 0.79	6.21 ± 1.90	4.85 ± 1.76	0.8507	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
∑PUFA	**45.00 ± 6.59**	**48.44 ± 5.83**	**56.02 ± 7.34**	**57.95 ± 14.07**	>0.9999	0.1208	0.0676	0.6951	0.4534	>0.9999

The first two axes of the PCO analysis explained 68.3% of the FA variation in the data set (PCO axis 1: 56.8%, PCO axis 2: 11.5%) (Figure 8). ANOSIM revealed significant differences among FA profiles of *C. edule* from different channels (p = 0.041) with the exception of specimens sampled in Ilhavo and Espinheiro (p = 0.155). The ANOSIM performed using FA classes (i.e. SFA, MUFA and PUFA) also showed significant differences among channels, apart from São Jacinto and Espinheiro (p = 0.059) and Ilhavo and Espinheiro (p = 0.296) for SFA, and Mira and Espinheiro Channels (p = 0.071) and Ilhavo and Espinheiro Channels (p = 0.179) for PUFA (Table 2).

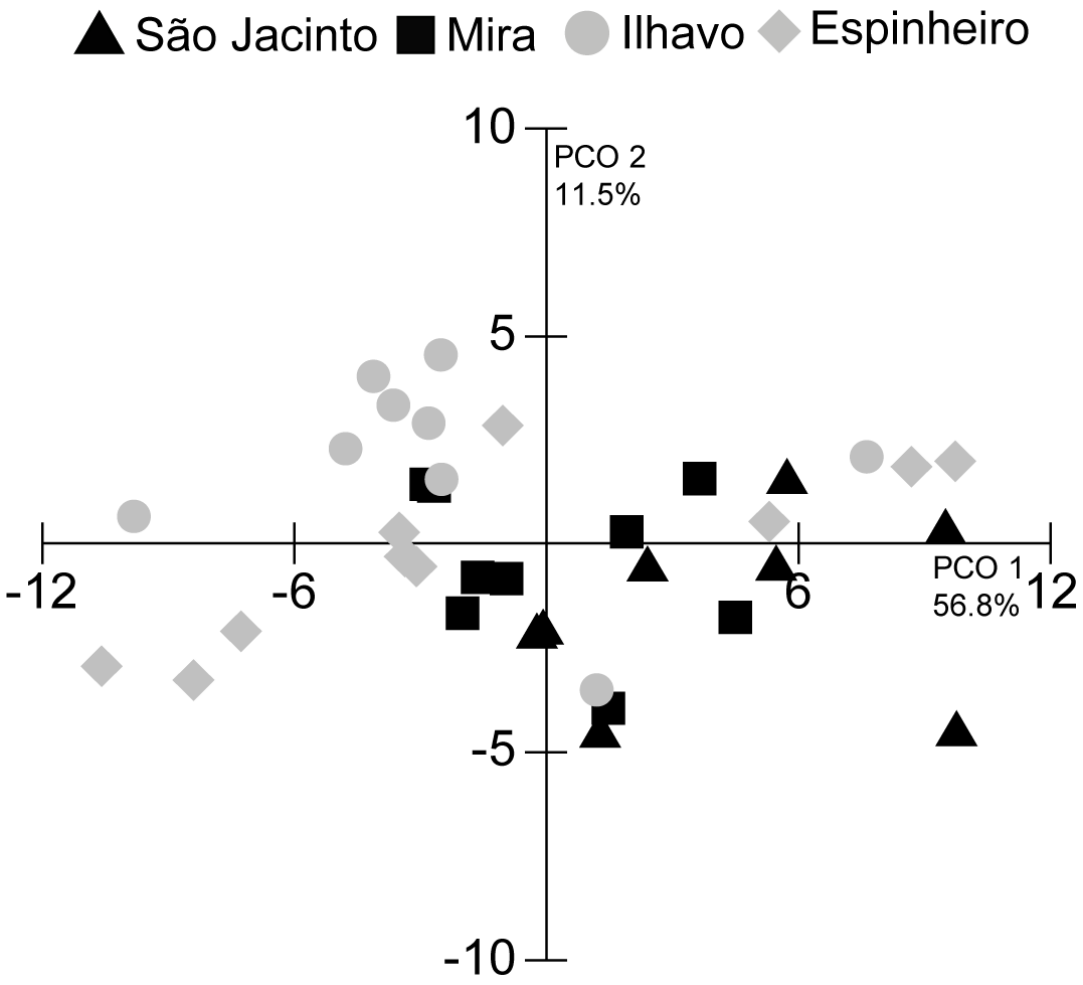

Figure 8. Principal coordinates analysis of the fatty acid composition of the adductor muscle of *Cerastoderma edule* from São Jacinto, Mira, Ilhavo and Espinheiro channels in Ria de Aveiro, Portugal

Table 2. Similarity values (ANOSIM) between all fatty acids (FA), saturated (SFA) and polyunsaturated (PUFA) fatty acids in the adductor muscle of *Cerastoderma edule* from São Jacinto, Mira, Ilhavo and Espinheiro channels in Ria de Aveiro, Portugal.

Channels	All FA		SFA		PUFA		MUFA	
	R	***p***	**R**	***p***	**R**	***p***	**R**	***P***
São Jacinto *vs* Mira	0.238	0.020	0.145	0.045	0.222	0.019	0.112	0.056
São Jacinto *vs* Ilhavo	0.358	0.006	0.428	0.002	0.362	0.004	0.046	0.231
São Jacinto *vs* Espinheiro	0.196	0.041	0.135	0.059	0.223	0.023	0.020	0.301
Mira *vs* Ilhavo	0.199	0.017	0.301	0.003	0.147	0.034	-0.010	0.455
Mira *vs* Espinheiro	0.154	0.041	0.182	0.010	0.105	0.071	0.034	0.261
Ilhavo *vs* Espinheiro	0.062	0.155	0.022	0.296	0.050	0.179	-0.009	0.402

SIMPER analysis (Table 3) revealed that PA and DHA were generally among the FA that most contributed for the differences recorded among channels (e.g. more than 22% of the differences recorded between São Jacinto and Espinheiro were explained by 16:0 and DHA). While specimens originating from Espinheiro and Ilhavo channels showed a relatively similar FA profile, the content of oleic acid (18:1n9c) in the AM of *C. edule* was notably different between these two locations (p = 0.0283, Table 1). SIMPER also revealed that myristic acid was responsible for almost 10% of all differences recorded between the pool of FA displayed by the AM of cockles collected in these two channels (Table 3).

Table 3. Similarity percentage analysis (SIMPER) identifying which fatty acids (FA) contribute to the differences recorded in the adductor muscle of *Cerastoderma edule* from São Jacinto, Mira, Ilhavo and Espinheiro channels in Ria de Aveiro, Portugal.

São Jacinto *vs* Mira			**São Jacinto *vs* Ilhavo**			**São Jacinto *vs* Espinheiro**			**Mira *vs* Ilhavo**			**Mira *vs* Espinheiro**			**Ilhavo *vs* Espinheiro**		
FA	**Ind (%)**	**Cum (%)**	**FA**	**Ind (%)**	**Cum (%)**	**FA**	**Ind (%)**	**Cum (%)**	**FA**	**Ind (%)**	**Cum (%)**	**FA**	**Ind (%)**	**Cum (%)**	**FA**	**Ind (%)**	**Cum (%)**
14:0	9.42	9.42	22:3*n*-6	9.69	9.69	16:0	11.53	11.53	22:3*n*-6	8.73	8.73	22:6*n*-3	10.31	10.31	14:0	9.30	9.30
22:4*n*-3	8.00	17.42	16:0	9.19	18.87	22:6*n*-3	10.71	22.24	14:0	8.70	17.43	16:0	9.87	20.18	22:6*n*-3	8.24	17.54
22:2*n*-9	7.65	25.07	22:4*n*-3	8.42	27.29	22:4*n*-3	8.79	31.03	22:2n9	7.57	25.00	14:0	8.86	29.04	16:0	8.17	25.71
16:0	7.65	32.72	14:0	8.35	35.64	22:2*n*-9	7.16	38.19	16:0	6.80	31.81	22:4*n*-3	7.66	36.70	22:2*n*-9	7.71	33.43
22:6*n*-3	7.27	39.99	22:2*n*-9	8.15	43.79	14:0	6.98	45.17	22:4*n*-6	6.70	38.51	16:1*n*-9	7.48	44.19	22:3*n*-6	7.70	41.12
22:3*n*-6	6.85	46.84	22:6*n*-3	7.17	50.96	16:1*n*-9	6.71	51.87	17:0	6.53	45.04	18:0	6.73	50.92	22:4*n*-3	7.03	48.16
16:1*n*-9	6.03	52.88	22:4*n*-6	5.55	56.51	22:3*n*-6	6.71	58.58	22:4*n*-3	6.23	51.28	22:2*n*-9	5.74	56.66	16:1*n*-9	7.01	55.17
15:0	5.68	58.56	15:0	5.18	61.69	18:0	5.47	64.05	22:6*n*-3	5.89	57.17	22:3*n*-6	5.43	62.08	18:0	5.31	60.48
20:1*n*-9	5.53	64.09	16:1*n*-9	4.61	66.30	18:3*n*-6	4.33	68.38	16:1*n*-9	5.53	62.70	17:0	5.40	67.49	22:4*n*-6	5.07	65.54
18:3*n*-6	5.00	69.09	18:3*n*-6	4.42	70.72	15:0	4.22	72.60	18:3*n*-6	4.99	67.69	18:3*n*-6	4.65	72.14	18:3*n*-6	4.80	70.34
18:1*n*-9t	4.51	73.60	18:1*n*-9t	4.14	74.85	18:1*n*-9t	3.99	76.59	15:0	4.21	71.89	21:5*n*-3	3.73	75.87	17:0	4.09	74.43
17:0	3.66	77.26	20:1*n*-9	3.73	78.58	17:0	3.62	80.20	21:5*n*-3	3.82	75.71	18:1*n*-9c	3.61	79.48	21:5*n*-3	3.71	78.14
21:5*n*-3	3.60	80.87	17:0	3.65	82.24				18:0	3.61	79.32	20:5*n*-3	3.21	82.69	20:3*n*-3	3.50	81.64
									20:3*n*-3	3.53	82.86						

2.1.4. Discussion

Most of the available studies on the FA profile of bivalves focus on the analysis of their whole body, gonads, gills or digestive gland (Nemova, Fokina, Nefedova, Ruokolainen, & Bakhmet, 2013; Pastoriza, Gallardo, Franco, & Sampedro, 1981; Perez et al., 2013). However, results from the latter two studies reveal notable variations of the FA profile with diet and environmental conditions (Nemova, Fokina, Nefedova, Ruokolainen, & Bakhmet, 2013; Pastoriza, Gallardo, Franco, & Sampedro, 1981). In order to minimize variability of FA profile associated with diet, this study solely analysed the FA content of the AM of *C. edule* (Delaporte et al., 2005). This approach was already successfully employed to discriminate bivalves originating from different locations (Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen, 2010; Olsen, Grahl-Nielsen, & Schander, 2009) and contrasting habitats (10 m vs. 31 m depth; Napolitano, Macdonald, Thompson, & Ackman, 1992). Moreover, Perez et al. (2013), combined FA analysis with stable isotopes to assign the location of the origin of bivalves (*Venus verrucosa*) with a spatial resolution < 10 km. The present study shows, for first time, that the FA profile of the AM alone holds to the potential to be used for geographical traceability of bivalves with a similar resolution (< 10 km).

The dominance of PUFA, followed by SFA and MUFA, in the FA profiles of the AM has already been recorded in other bivalve species, such as the fan mussel *Pinna nobilis* (Najdek, Blažina, Ezgeta-Balić, & Peharda, 2013), the scallops *Pecten maximus* (Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen, 2010,) and *Placopecten magellanicus* (Napolitano, Macdonald, Thompson, & Ackman, 1992; Napolitano, Pollero, Gayoso, Macdonald, & Thompson, 1997), in *Astarte sulcate* (Olsen, Grahl-Nielsen, & Schander, 2009), the flat oyster *Ostrea edulis*, the black mussel *Mytilus galloprovincialis*, the bearded horse mussel *Modiolus barbatus* and Noah's ark shell *Arca noae* (Ezgeta-Balić, Najdek, Peharda, & Blažina, 2012). In general, and as in the present study, all these works revealed that the dominant SFA was PA, followed by EPA and DHA as the most abundant PUFA (see Galap, NetchitaïLo, Leboulenger, & Grillot, 1999). PA and DHA were also responsible for most of the differences recorded among Ria de Aveiro channels (Table 1). Both PA and DHA showed significant shifts in their relative abundance with geographical location, which is likely associated with a differential physiological response to variable environmental conditions. Similarities in the FA profile of cockles between São Jacinto and Mira, as well as between Ilhavo and Espinheiro (Table 1; Table S1 on appendix B), were likely associated with the geographical proximity among these channels and their similar environmental conditions. Despite the major axis of variation (axis 1; Figure 8) did not clearly separate the specimens originating from each of the four channels of the coastal lagoon, specimens from Ilhavo and Espinheiro channels were mostly separated, which contrasted with specimens from São Jacinto and Mira channels that were relatively spread throughout the PCO. It is worth noting that specimens originating from areas closer to the inlet (São Jacinto and Mira) are likely to be less exposed to lower salinities (Table S1 on appendix B) than those originating from

channels located more upstream (Espinheiro and Ilhavo) (Figure 8). Bivalves further away from the inlet may experience a sharper decrease in salinity during rainfall, due to a notable freshwater contribution of small rivers and streams bordering Ria de Aveiro, and a higher increase in salinity during the summertime promoted by a lower water exchange due to the distance from the inlet and consequent increase in evaporation. Bivalves exposed to higher saline fluctuations are also expected to display a decrease in their levels of SFA, which is responsible to stabilize the bilayer structure of cell membranes, and an increase in their concentration of PUFA to enhance bilayer fluidity (Nemova, Fokina, Nefedova, Ruokolainen, & Bakhmet, 2013).

The structural nature of the lipids presents in the AM of bivalves (mostly phospholipids and sterols) provides a stable FA signature that is primarily determined by environmental conditions and functions of the cellular membrane rather than dietary regimes (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003; Napolitano, Pollero, Gayoso, Macdonald, & Thompson, 1997). At present, most studies available on the FA profiles of bivalves are focused on the analysis of the whole body or other organs than the AM (e.g. gonads, gills and digestive gland; Napolitano, Macdonald, Thompson, & Ackman, 1992; Perez et al., 2013) that are regulated by intrinsic (e.g. age, phylogeny and sex) and external (e.g. diet, salinity and depth) factors (Olsen, Grahl-Nielsen, & Schander, 2009). Conversely, the FA signature of the AM is less prone to fast and dramatic shifts as compared to other organs (e.g. gonads and the digestive gland; Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003; Napolitano, Macdonald, Thompson, & Ackman, 1992). As this study was carried out in Ria de Aveiro, which is a system with a remarkable spatial variability of environmental conditions among different channels (Dias, Lopes, & Dekeyser, 1999), particularly saline fluctuations, it is likely that environmental variability plays a major role on the FA signature of the AM of cockles from different channels of this ecosystem (see supplementary information).

It is important to note that the FA profile of bivalves may also exhibit temporal variability associated with environmental conditions (Birkely, Grahl-Nielsen, & Gulliksen, 2003; Ezgeta-Balić, Najdek, Peharda, & Blažina, 2012). While such temporal variability may be seen as a potential obstacle for using this approach for traceability purposes, it can be circumvented by authorities. For instance, the FA profiles of the AM of control samples collected from the capture/production area claimed as place of origin by the bivalve trader can be matched with those from the batch of bivalves being surveyed. If significant differences are observed, it is likely that the place of origin of the bivalves being traded is not the one claimed by the trader. The average shelf life of fresh cockles traded is generally $\leq$ 5 days after harvesting. Therefore, given this limited shelf life of fresh bivalves, it is unlikely to find significant differences in the FA profiles of the AM of control samples and the batch of bivalves being traded caused by the time frame between the collection of both (always $\leq$ 5 days). Although the dynamics of the FA profile of cockles during ice storage has never been assessed, a study performed on the whole body of blue

mussels *M. edulis* revealed no major shifts on the FA profile during a 14-day storage period in ice (Khan, Parrish, & Shahidi, 2005). Therefore, while it is likely that the FA profile of *C. edule* may remain stable during a 5-day storage period in ice, this assumption should be tested in the future, specifically for the adductor muscle of *C. edule*.

In conclusion, our null hypothesis that no significant differences in the FA profile of the AM of *C. edule* was expected between specimens captured in different channels of Ria de Aveiro was rejected. Indeed, this biochemical approach allowed us to differentiate cockles originating from different channels of this coastal lagoon. The spatial resolution achieved with this methodological approach revealed the ability to discriminate capture/production areas for bivalves classified as "B" and "C" within the same coastal system (e.g. São Jacinto and Ilhavo). However, this approach was unable to discriminate between areas classified as "C" (Ilhavo and Espinheiro). These findings are important to guarantee that specimens are not mislabelled and illegally traded, as well as address current legislation on seafood traceability (Leal, Pimentel, Ricardo, Rosa, & Calado, 2015). Additionally, geographical traceability may play a key role for fishermen/producers willing to differentiate and add value to their products by assuring that bivalves being traded originate from regions that may be microbiologically safer than others (e.g. displaying lower loads of *Vibrio* spp. or other microorganisms of concern for public health). While this approach is relatively low-cost, it can be further simplified, and performed in a faster way, by employing a direct esterification of the AM followed by extraction (Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen., 2010; Olsen, Grahl-Nielsen, & Schander, 2009). Future studies should try to apply this methodology to other commercially important bivalves, as well as monitor seasonal and interannual variability to ascertain the suitability of assembling a database for tracing their place of origin.

CHAPTER 2. THE USE OF FATTY ACID PROFILES IN BIVALVES TRACEABILITY

2.2. Spatio-temporal variability in the fatty acid profile of the adductor muscle of the common cockle *Cerastoderma edule* and its relevance for tracing geographic origin

The material & methods, results and discussion presented in this section were integrally published as follow:

Fernando Ricardo, M. Rosário Domingues & Ricardo Calado (2017). Spatio-temporal variability in the fatty acid profile of the adductor muscle of the common cockle *Cerastoderma edule* and its relevance for tracing geographic origin. *Food Control 81,* 173-180.

2.2.1. Background and aim of the study

Along the Portuguese coast, common cockle (*Cerastoderma edule*) is the most commonly harvested bivalve with an increasing trend in recent years (INE, 2015, 2014).

FA are considered effective biomarkers of certain groups of organisms (e.g. bacteria, diatoms and dinoflagellates) able to provide information about the nutritional value and their geographic origin. Therefore, the present study aimed to characterize in which way the spatial distribution of *C. edule* among eight ecosystems along the Portuguese coast (Ria de Aveiro, Óbidos lagoon, Tagus estuary, Albufeira lagoon, Sado estuary, Mira estuary, Ria do Alvor e Ria Formosa) affects the FA profiles of the AM of this species. This knowledge could also be useful in the assessment of the FA profiles of the AM potential for the successfully discriminate their geographic origin. In this way, it is of great relevance to evaluate the temporal variability. Thus, this study also aimed to determine the temporal variability of FA profile between two consecutive years in areas within the same ecosystem.

2.2.2.Material and methods

2.2.2.1. Study area and sample collection

A total of 100 samples of *C. edule* with a shell length > 25 mm (commercial size, approximately 3 years old) (Seed & Brown, 1978) were collected during June-July of 2014 from eight different Portuguese ecosystems where this species is commercially explored: Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria do Alvor (RAl) e Ria Formosa (RF) (Figure 9). In RAv three of the main channels (Mira, Ílhavo and Espinheiro) of this coastal lagoon were sampled. Two areas were surveyed in each channel (Figure 9a) and five replicates were collected per area (3 channels X 2 areas X 5 replicates = 30 samples). For OL, TE, AL, SE and RF two areas were surveyed with five cockles being collected per area (5 ecosystems X 2 areas X 5 replicates = 50 samples). In RAl and ME due to few abundance of cockles, were collected ten specimens in just one area (2 ecosystems X 1 areas X 10 replicates = 20 samples).

In order to evaluate the potential existence of temporal variability in the FA profile of the AM, specimens of *C. edule* from Ílhavo (I) and Espinheiro (E) channels at RAv were collected exactly in the same locations in June 2013 and June-July 2014 for comparison. Five specimens were collected per area in these two consecutive years (1 ecosystem X 2 channels X 2 areas X 2 years X 5 replicates = 40 samples). Bivalves were surveyed during the summer time, as the consumption of this highly priced seafood significantly increases during this period and thus with increasing demand and higher market values fraudulent practices are more prone to occur.

All samples were collected manually with the help of a hand-rake and stored in aseptic plastic bags. After collection, cockles were kept refrigerated and transported to the laboratory and

stored at -20 °C for further processing. All collected specimens were dissected to extract the AM, which were freeze-dried then stored at − 80 °C for subsequent FA analysis.

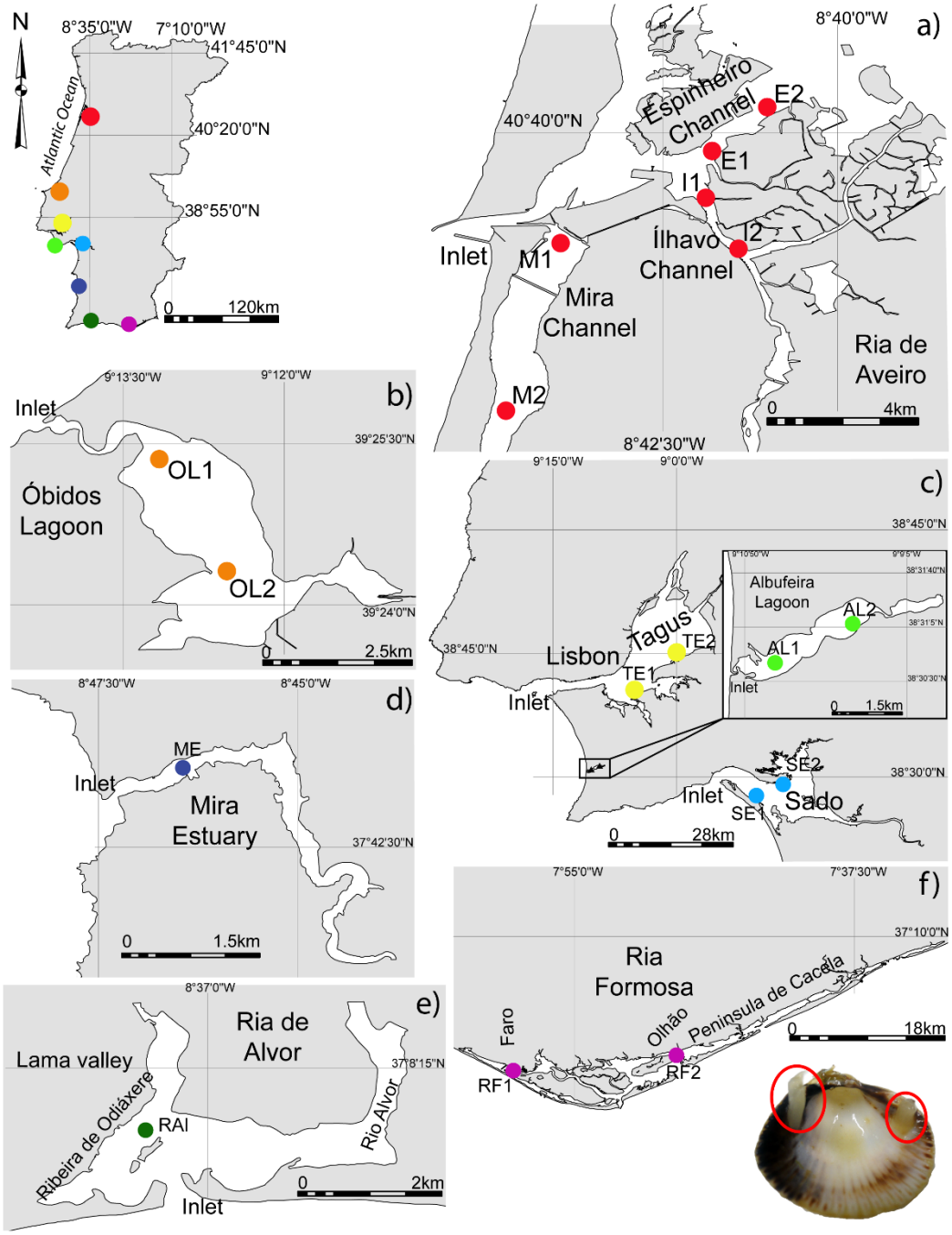

Figure 9. Sampling locations of *Cerastoderma edule* in mainland Portugal: a) Ria de Aveiro (RAv; M1:40°38'26.30"N, 8°43'58.90"W; M2: 40°35'58.30"N, 8°44'47.80"W; I1: 40°38′ 22.36″ N, 8°41′ 24.93″ W, I2: 40°37′ 03.10″ N, 8°40′ 48.00″ W, E1: 40°39′ 48.50″ N, 8°41′ 45.03″ W and E2: 40°40′ 37.10″ N, 8°40′ 28.90″ W), b) Óbidos lagoon (OL1: 39°25'20.34"N, 9°13'14.54"W and OL2: 39°24'2.01"N, 9°12'30.91"W), c) Tagus estuary (TE1: 38°39'27.44"N, 9°6'35.95"W and TE2: 38°44'5.18"N, 9°0'46.54"W), Albufeira lagoon (AL1: 38°30'36.67"N, 9°10'32.96"W and AL2: 38°31'1.33"N, 9°9'53.16"W) and Sado estuary (SE1: 38°27'46.00"N, 8°51'32.00"W and SE2: 38°29'13.25"N, 8°48'52.79"W) d) Mira estuary (ME: 37°43'30.60"N, 8°46'15.40"W), e) Ria de Alvor (RAl: 37°07'55.7"N, 8°37'27.40"W) and f) Ria Formosa (RF1: 37°00'23.20"N, 7°59'28.40"W and 37°01'24.30"N, 7°49'49.50"W). The map was created using the software ArcGIS v10.2.2.

2.2.2.2. Fatty acids analysis

Total lipids from the AM of each individual cockle were quantified through gravimetric method after extraction following the procedure described by Bligh & Dyer (1959). Methyl esters of FA (FAME) were prepared according to Aued-Pimentel, Lago, Chaves, & Kumagai (2004) method (by transmethylation of FA using a mixture of methanolic solution KOH (2 M) and satured NaCl. Gas chromatography–mass spectrometry (GC–MS) analysis were performed using an Agilent Technologies 6890 N Network (Santa Clara, CA) equipped with a DB-FFAP column with 30 m of length, 0.32 mm of internal diameter, and 0.25 μm of film thickness (J&W Scientific, Folsom, CA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C, with a linear increase to 220 °C being performed at 14.4 °C min^{-1}, followed by a linear increase at 10 °C min^{-1} to 240 °C and 5 °C min^{-1} to 250 °C. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as the carrier gas at a flow rate of 0.5 mL min^{-1}. Individual FA peaks were identified by comparing the retention time and mass spectra of each FA relative to 34 mixed FA standards (C4-C24, Supelco 37 Component Fame Mix), and confirmed by comparison with the spectral library "The AOCS Lipid Library" (AOCS, 2012).

2.2.2.3. Statistical analysis

In order to select the best subset of variables that may explain potential differences between the different areas sampled within each ecosystem, FA were grouped by classes as saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA). Within each class, FA displaying the same pattern were pooled to simplify further statistical analysis. A preliminary multivariate analysis of variance (MANOVA; Table S3 on appendix C) was performed to detect significant differences in the FA profile displayed by the AM of *C. edule* sampled in different areas of the same ecosystem. As no significant differences were recorded between areas within the same ecosystem, samples from each ecosystem were grouped resulting in a total of 10 replicates per ecosystem.

Differences in the FA profile of the AM of *C. edule* from the eight ecosystems surveyed were analyzed through a MANOVA. One-way analysis of variance (ANOVA) was also used to detected differences among ecosystems for each individual FA and post hoc Tukey test was used whenever ANOVA results revealed the existence of significant differences ($p < 0.05$). Moreover, a linear discriminant analysis (LDA) was used to test the possibility of successfully discriminating the geographic origin of sampled specimens through the FA profile of their AM. Concerning the potential existence of temporal variability of the FA profile displayed by the AM of common

cockles over the two consecutive years that were surveyed in RAv, FA were grouped by classes before MANOVA analysis and subsequent statistical tests were performed as already described above. Normality and variance homogeneity (Pillai Trace test) were tested and data was transformed (log X+1). All statistical analyses were performed using R (R Development Core Team, 2015).

2.2.3. Results

The FA profile of the AM of *C. edule* from different ecosystems is shown in Figure 10 and Table S4 on appendix C. Twenty-one FA were identified; SFA represented 26-31% of the total pool of FA, MUFA represented 12-19% and PUFA represented the majority of FA by ranging from 50% to 59% of the whole pool. The most representative SFA were palmitic (16:0) and stearic (18:0) acids, while the dominant MUFA were elaidic (18:1*n*-9) and eicosenoic acid (20:1*n*-9/11) (Figure 10). The most abundant PUFA were eicosapentaenoic (20:5*n*-3; EPA) and docosahexaenoic (22:6*n*-3; DHA) acids, which together represented at least 60% of all PUFA recorded in the AM of common cockles and varied significantly ($p < 0.05$; Figure S1a-d, Tukey´s HSD, $p < 0.05$) among specimens from different ecosystems.

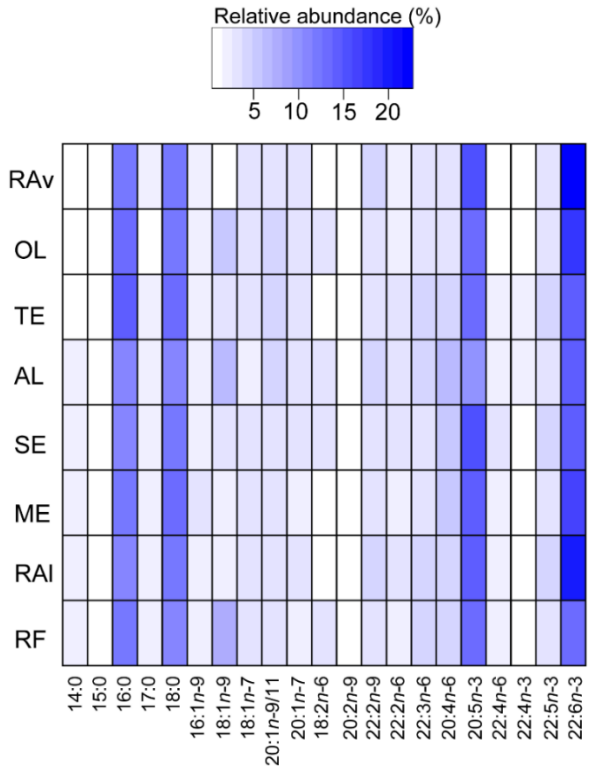

Figure 10. Heatmap representing the relative abundances (%) of fatty acids in the adductor muscle of live common cockles *Cerastoderma edule* from eight ecosystems along the Portuguese coast: Ria de Aveiro (RAv), Óbidos Lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

The MANOVA performed to investigate potential differences on the FA profiles of the AM of *C. edule* from different geographic origins revealed significant levels of variability among the eight ecosystems surveyed during the present work ($F = 6.47$, $p < 0.0001$). Considering each FA individually, 22:3*n*-6 was the sole FA which did not display any significant difference among ecosystems ($p > 0.05$). Specimens from RAv, RAl and OL recorded higher levels of total *n*-3 FA and lower levels of total *n*-6 FA that was reflected in higher *n*-3/*n*-6 ratios (3.75 for RAv, 2.77 for RAl and 2.68 for OL). Nevertheless, significant differences in *n*-3/*n*-6 ratio were only recorded between RAv and the other ecosystems sampled ($p < 0.05$; Figure S1a). AL presented significantly lower levels of EPA ($p < 0.05$; Figure S1b) comparatively to the other ecosystems. The FA that most contributed for the differences recorded among TE, AL, SE and ME and the other ecosystem surveyed were pentadecylic (15:0), margaric (17:0) (Figure S1c), 18:1*n*-7 and arachidonic acid (20:4*n*-6) ($p < 0.05$; Figure S1d). Specimens from RF showed higher levels of MUFA, namely 18:1*n*-9, being significantly different from those recorded in other ecosystems ($p < 0.05$; Figure S1d). The first three discriminant functions of the LDA analysis explained 78.9% of all FA profiles variation (LDA 1: 44.1%, LDA 2: 20.9% and LDA 3: 13.9%), with results revealing an overall accuracy of 100% (Figure 11 and Table 4).

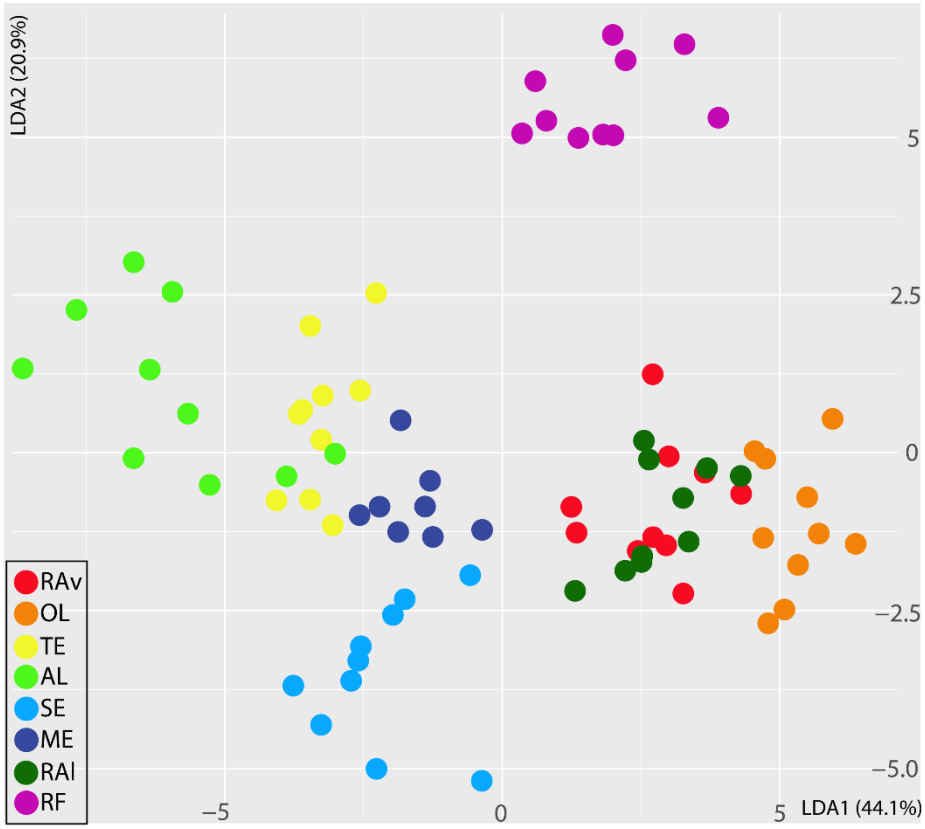

Figure 11. Linear discriminant analysis (LDA) of cockles based on trace elements fingerprints of shells collected from eight different ecosystems along the Portuguese coast: Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

Table 4. Classification success (by ecosystem) of a linear discriminant analysis (LDA) based on fatty acid profile of the adductor muscle of *Cerastoderma edule*. Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

	% Predicted Ecosystem								Total per ecosystem	% correct (ecosystem)
	RAv	OL	TE	AL	SE	ME	RAl	RF		
Original Ecosystem										
RAv	100	0	0	0	0	0	0	0	10	100
OL	0	100	0	0	0	0	0	0	10	100
TE	0	0	100	0	0	0	0	0	10	100
AL	0	0	0	100	0	0	0	0	10	100
SE	0	0	0	0	100	0	0	0	10	100
ME	0	0	0	0	0	100	0	0	10	100
RAl	0	0	0	0	0	0	100	0	10	100
RF	0	0	0	0	0	0	0	100	10	100
Average classification success										100

The interaction term [years x areas] revealed the existence of significant differences (MANOVA, $F = 2.73$, $p < 0.0001$) and the analysis of FA profiles of the AM of *C. edule* between years was made separately for each sampled area, considering a total of seven different groups of FA (16:0, 18:0, 14:0+15:0+17:0, MUFA, EPA, DHA and remaining PUFA). An increase in the DHA and remaining PUFA groups, along with a decrease in 16:0, 18:0, MUFA and EPA groups were observed from 2013 to 2014 in the specimens collected channels I and E of RAv (Figure 12). Contrarily, in channel E, the relative abundance of FA group 14:0+15:0+17:0 decreased in channel I from 2013 to 2014 (Figure 12). ANOVA analysis among FA groups showed that MUFA, EPA and PUFA varied significantly between 2013 and 2014 in both channels. The 16:0 group also showed significant differences between years in channel E, while channel I recorded significant difference for the FA group 14:0+15:0+17:0 (Figure 12). In 2013, 16:0, PUFA and DHA showed significant differences between channels, while in 2014, 16:0 was the only FA group that revealed significant differences between channels.

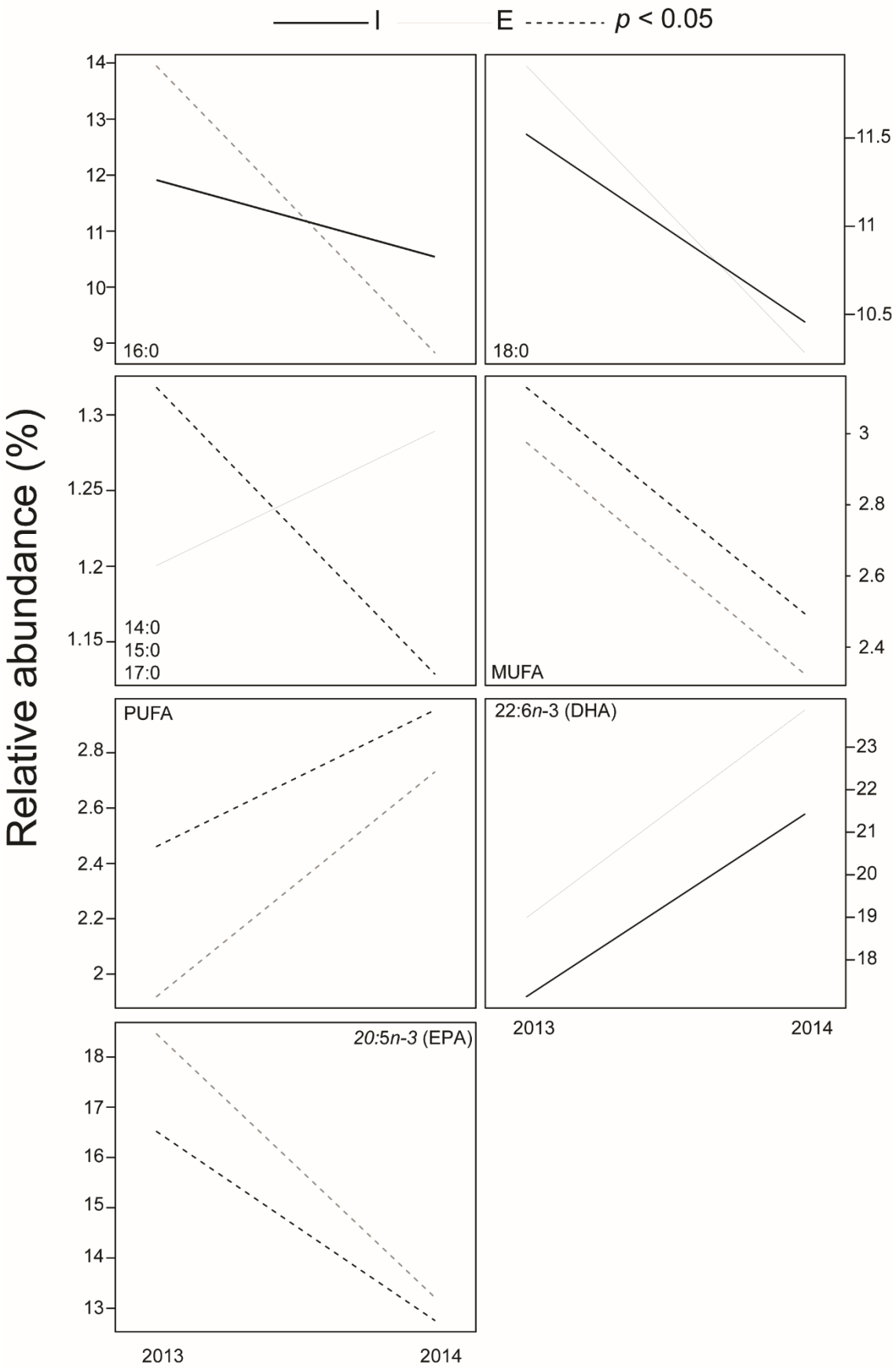

Figure 12. Evolution of fatty acid profile groups of the adductor muscle of *Cerastoderma edule* from 2013 to 2014 in areas: Ílhavo Channel (I) and Espinheiro Channel (E). The dotted lines represent significant differences in the fatty acids profiles between years ($p < 0.05$).

2.2.4. Discussion

The significant differences recorded in the FA profile of the AM of cockles collected from different ecosystem revealed that this approach can be used to trace their place of origin with a high level of certainty (100% success according to LDA results, see Table 4). Specimens collected in AL displayed the lowest levels of EPA when compared to conspecifics sampled in all other ecosystems surveyed in the present work. The abundance of EPA in FA profiles is directly related with the consumption of microalgae (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003). This finding suggests that common cockles in AL may be exposed to less favourable trophic scenarios, likely a consequence of the intermittent closure of the inlet of this coastal lagoon (Fortunato et al., 2014). This closure promotes dramatic shifts in the water mass of AL and may favour blooms of phytoplankton species with higher affinities to brackish-low salinity conditions that commonly display lower levels of EPA in their FA profiles (Coutinho, Brito, Pereira, Gonçalves, & Moita, 2012). Concerning the AM of specimens originating from TE, AL, SE and ME, these were characterized by displaying high levels of 15:0, 17:0 and 18:1*n*-7. These FA are often present in bivalves tissues due to the ingestion of bacterioplankton (Bergé & Barnathan, 2005). High levels of these FA in the AM of *C. edule* likely reflect potential sources of faecal contamination in bivalve harvesting areas. It is therefore possible that water runoffs, livestock production, sewer overflows and the presence of recreational ports and shipyards nearby sampling locations may contribute to the occurrence of bacterioplankton whose FA profile is fingerprinted in the AM (Anacleto et al., 2013; Coutinho, Brito, Pereira, Gonçalves, & Moita, 2012; Vasconcelos et al., 2007). Concerning the significantly higher levels of 20:4*n*-6 present in AM of cockles originating from TE, AL, SE and ME, it is important to highlight that such FA fingerprint is commonly recorded in diatom dominated locations (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003), which often reflect intensive anthropogenic presence (as known to occur in these study locations). The AM of specimens from RF exhibited the highest levels of 18:1*n*-9 and 20:1*n*-9/11, two trophic markers associated with the ingestion of zooplankton (Maloy, Culloty, & Slater, 2009). Common cockle specimens sampled at OL, RAl and mainly at RAv, presented high *n*-3/*n*-6 ratios in the FA profiles of their AM. RAl and RAv are two of the main bivalve producing areas in mainland Portugal (Leite, Afonso, & Cancela, 2004; Vale, Canário, Caetano, Lavrado, & Brito, 2008) and are well known for displaying highly favourable trophic conditions for the grow-out of bivalves. In other words, favourable trophic conditions likely correspond to marine phytoplankton blooms with *n*-3 PUFA prevailing in their FA pool. The FA profile of the AM of *C. edule*, namely for specimens in RAv, may also be due to the prevalence of upwelling conditions in this areas (Alfaro, Hernández, Le Marc, & Pin, 2013; Alvarez et al., 2013), which favours phytoplankton blooms.

As the present study was performed along a latitudinal gradient, it could be anticipated that specimens occurring in ecosystems experiencing lower water temperatures would display a

higher level of FA unsaturation, as this feature allows cellular membranes to maintain their fluidity without compromising homeostasis between intracellular and extracellular environments (Copeman & Parrish, 2004; Napolitano, Macdonald, Thompson, & Ackman., 1992). This prediction was confirmed in our study, as common cockles sampled in ecosystems located in the southern coast of mainland Portugal, thus experiencing higher temperatures, displayed a lower level of unsaturation than northern ecosystems. As referred above, FA profiles of the AM are primarily determined by environmental conditions and functions of the cellular membrane, rather than short term shifts in dietary regimes (Dalsgaard, St John, Kattner, Müller-Navarra, & Hagen, 2003; Napolitano, Pollero, Gayoso, Macdonald, & Thompson, 1997). These features are paramount for the selection of the AM as the target matrix to be monitored for the purpose of tracing the geographic origin of bivalves. While other bivalve organs display a high metabolic activity, such as gonads and the digestive gland, and are more prone to be influenced by recent dietary items, the AM exhibits a lower turnover rate of FA and rather reflects the "average" feeding regime experienced over longer periods of time (Paulet, Lorrain, Richard, & Pouvreau, 2006).

Some of the ecosystems surveyed in our study display high spatial variability on their environmental conditions, with the conditions experienced by *C. edule* in RAv in particular being remarkably variable among the different channels of this coastal lagoon (Dias, Lopes, & Dekeyser, 1999). In order to maintain homeostasis between intracellular and extracellular environments, marine organisms need to adjust physiologically to such variable environmental conditions, from short term shifts promoted by tidal rhythms to longer term shifts at seasonal or annual time scales (Nemova, Fokina, Nefedova, Ruokolainen, & Bakhmet, 2013). These long physiological adaptations area perceptible in the significant shifts in displayed the FA profiles of the AM of cockles sampled in the two different channels of RAv over two consecutive years. Several studies have already reported the existence of temporal variability in the FA profile of the AM of bivalves. Ezgeta-Balić, Najdek, Peharda, & Blažina et al. (2012) reported significant temporal variability (two-month period) in four commercially important bivalve species in the eastern Adriatic Sea (Mali Ston Bay, Croatia), while Puccinelli, McQuaid, & Noyon (2016) also recorded inter-seasonal variability under two mesoscale nearshore oceanographic conditions (upwelling and non-upwelling) in the FA profiles of the AM of mussels (*Mytilus galloprovinciallis*) in west coast of South Africa.

Overall, the present study shows that the FA profile displayed by the AM of *C. edule* can be successfully used with a high level of accuracy to trace their geographic origin over a range of ecosystems distributed along a latitudinal gradient. These findings reinforce the claims by Ricardo et al. (2015a), that advocated the use of this biochemical approach to put into practice a traceability framework that can allow the verification of the geographic origin of claimed by traders of fresh bivalves. The existence of temporal variability advises caution on the use of

previously available information on the FA profile of the AM of bivalves for traceability purposes, as significant shifts do occur and they may lower the accuracy of the analysis by assigning specimens to erroneous locations.

CHAPTER 2. THE USE OF FATTY ACID PROFILES IN BIVALVES TRACEABILITY

2.3. Fatty acid dynamics of the adductor muscle of live cockles (*Cerastoderma edule*) during their shelf-life and its relevance for traceability of geographic origin

The material & methods, results and discussion presented in this section were integrally published as follow:

Fernando Ricardo, Tânia Pimentel, Elisabete Maciel, Ana S.P. Moreira, M. Rosário Domingues & Ricardo Calado (2017). Fatty acid dynamics of the adductor muscle of live cockles (*Cerastoderma edule*) during their shelf-life and its relevance for traceability of geographic origin. *Food Control*, *77*, 192-198.

2.3.1. Background and aim of the study

Cockles (*Cerastoderma edule*) are commercially important bivalves that support several fisheries in European waters. The fatty acid (FA) profile of the adductor muscle (AM) of freshly collected live cockles can be used to reliably confirm their geographic origin. This approach is paramount for traceability, expose fraud and ensure food safety. However, no study has ever addressed if the FA profile of the AM of live cockles remains stable during shelf-life, as significant shifts may blur FA signatures recorded at harvest. The present study aimed to determine if the FA profile of the AM of live cockles (*C. edule*) displays any significant shifts during the shelf-life of this commercially important species and how long post-harvest can these FA profiles be used to reliably trace the geographic origin of these commercially important bivalves.

2.3.2. Material and methods

2.3.2.1. Study area and sample collection

Fresh cockles *C. edule* (*n* = 80) were collected by hand-raking in Mira Channel (Ria de Aveiro, Portugal; 40°36′ 39.50″ N, 8°44′ 47.40″ W), one of the most important commercial fishing areas for this species in mainland Portugal. All samples collected were immediately stored in aseptic bags and transported to the laboratory within approximately 30 min post-harvest. Packs of ten cockles were placed in mesh-bags and kept in a cold room at 4 ºC (Figure 13) during seven consecutive days. From the 10 individuals of each mesh-bag were randomly sampled 5 individuals at times T0 (sampling day), T1 (one day post-harvest), T2, T3, T4, T5, T6 and T7 (seven days post-harvest) (1 sampling area X 8 time points X 5 replicates = 40 samples). The adductor muscle (AM) from each cockle specimen was dissected using a sterilized scalpel and stored at -80 ºC until FA analysis. The rationale supporting the time frame of the present study (seven days post-harvest) was based upon a preliminary survey performed on five large retail surfaces trading live cockles that consider that the average shelf-life of live bivalves is of only 5 days. By employing a seven days' time frame, it could be expected that spoilage would occur and major shifts in the FA of the AM of live cockles could be recorded.

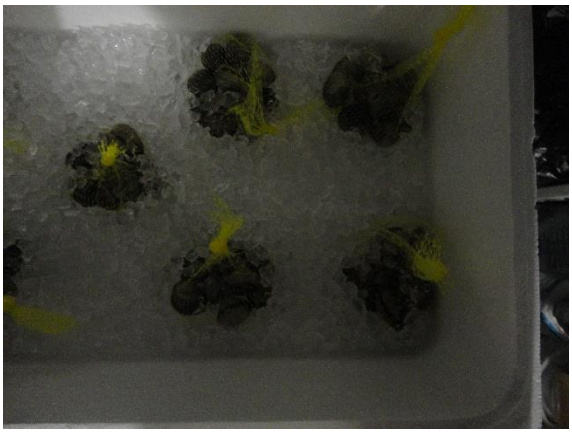

Figure 13. Packs of ten cockles were placed in mesh-bags and kept in a cold room at 4 ºC.

2.3.2.2. Fatty acids analysis

The extraction of total lipids of the AM of each individual cockle was performed according to the Bligh & Dyer (1959) method using methanol/chloroform (2:1, v/v). As phospholipids (PLs) are the main lipids present in the AM of bivalves (Napolitano, Pollero, Gayoso, Macdonald, & Thompson, 1997), PL were estimated through the phosphorus assay (Bartlett & Lewis, 1970) and an amount of each lipid extract containing 15 μg of PLs was used in the preparation of fatty acid methyl esters (FAMEs) following the procedure described by Aued-Pimentel, Lago, Chaves, & Kumagai (2004). The resulting FAMEs were dissolved in n-hexane (30 μL) and 4 μL of this solution was analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890 N Network (Santa Clara, CA) equipped with a DB-FFAP column with 30 m of length, 0.25 mm of internal diameter, and 0.32 μm of film thickness (J&W Scientific, Folsom, CA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C, with a linear increase to 220 °C at 14.4 °C min^{-1}, followed by linear increase at 10 °C min^{-1} to 240 °C, then at 5 °C min^{-1} to 250 °C. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 mL min^{-1}. Individual FA peaks were identified according to the methodology previously described by Ricardo et al. (2015a).

2.3.2.3. Statistical analysis

Biochemical data were expressed as the percentage of relative abundance of each FA recorded in each day. For a better understanding of our results, FA classes were separated as follows: saturated FA (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA), and highlyunsaturated FA (HUFA). It is worth noting that, in general, all FA with 2 ≥ double bonds are termed as PUFA; however, in the present study, we distinguished between PUFA (FA with 2 or 3 double bonds) and HUFA (FA with 4 ≥ double bonds). For statistical analysis, FA with the same pattern along the study period were grouped in order to simplify the analysis.

An analysis of multivariate (MANOVA) was performed to detect differences in the FA profiles of the AM of *C. edule* sampled along different shelf-life times. One-way analysis of variance (ANOVA) was used to assess differences between shelf-life times for each class of FA. The post-hoc Tukey test was used whenever the ANOVAs revealed the existence of significant differences ($p < 0.05$). A linear discriminant analysis (LDA) was performed to evaluate the potential use of FA profiles of the AM of cockles to discriminate between different shelf-life times and reveal the persistence of certain FA post-harvest.

All analyses were performed using (log x+1) transformed data in order to meet the multivariate normality and homoscedasticity (Pillai Trace test) required for MANOVA, as well as meet the normality and homogeneity of variance of ANOVA. Statistical analysis were performed using R (R Development Core Team, 2015).

2.3.3. Results

The average FA composition recorded for the AM of *C. edule* along its shelf-life is presented in Figure 14 and Table S5 (see appendix D). From the twenty-one FA identified, SFA represented 27-32%, MUFA 9-15% and PUFA 12-16%. HUFA were the most significant class of FA recorded in the bivalves surveyed, with their levels ranging between 39 and 45% of the total pool of FA. The main SFA were palmitic (16:0) and stearic acid (18:0), which represented more than 70% of all SFA recorded in the AM of cockles. The major MUFA was eicosenoic acid (20:1*n*-9), while the dominant PUFA were 22:2*n*-9 and 22:3*n*-6 with a similar contribution to the total pool of FA in the AM of cockles during their shelf-life. Concerning HUFA, the most abundant were eicosapentaenoic (EPA) (20:5*n*-3) and docosahexaenoic acid (DHA) (22:6*n*-3).

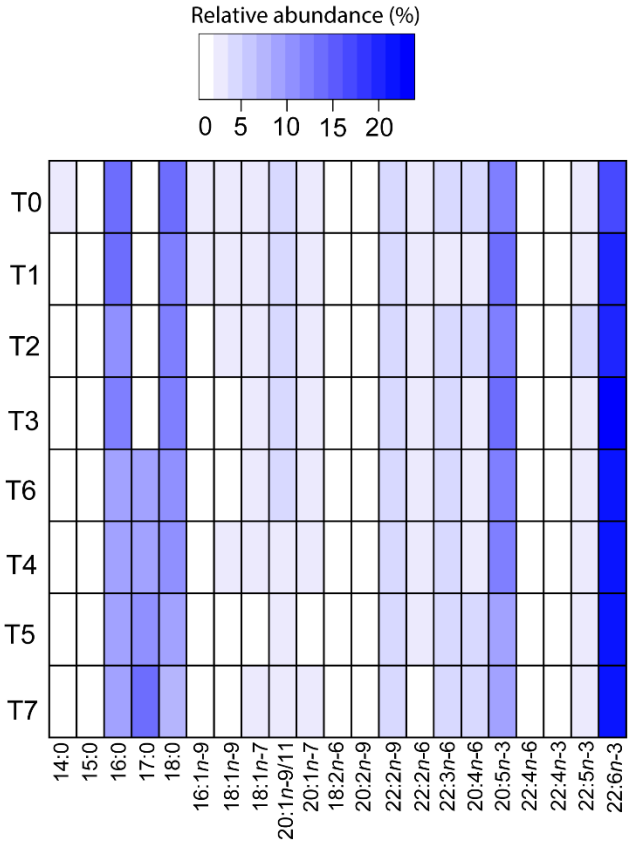

Figure 14. Heatmap representing the relative abundances (%) of fatty acids in the adductor muscle of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC.

The variability of SFA, MUFA, PUFA and HUFA displayed by the AM during the shelf-life of cockles is presented in Figure 15. PUFA and MUFA did not show any major fluctuations, with similar FA abundances being recorded during the study period. However, SFA and HUFA showed a different pattern along cockle's shelf-life time, with a decrease in 16:0 and 18:0 abundance and an increase of heptadecanoic acid (17:0), EPA and DHA.

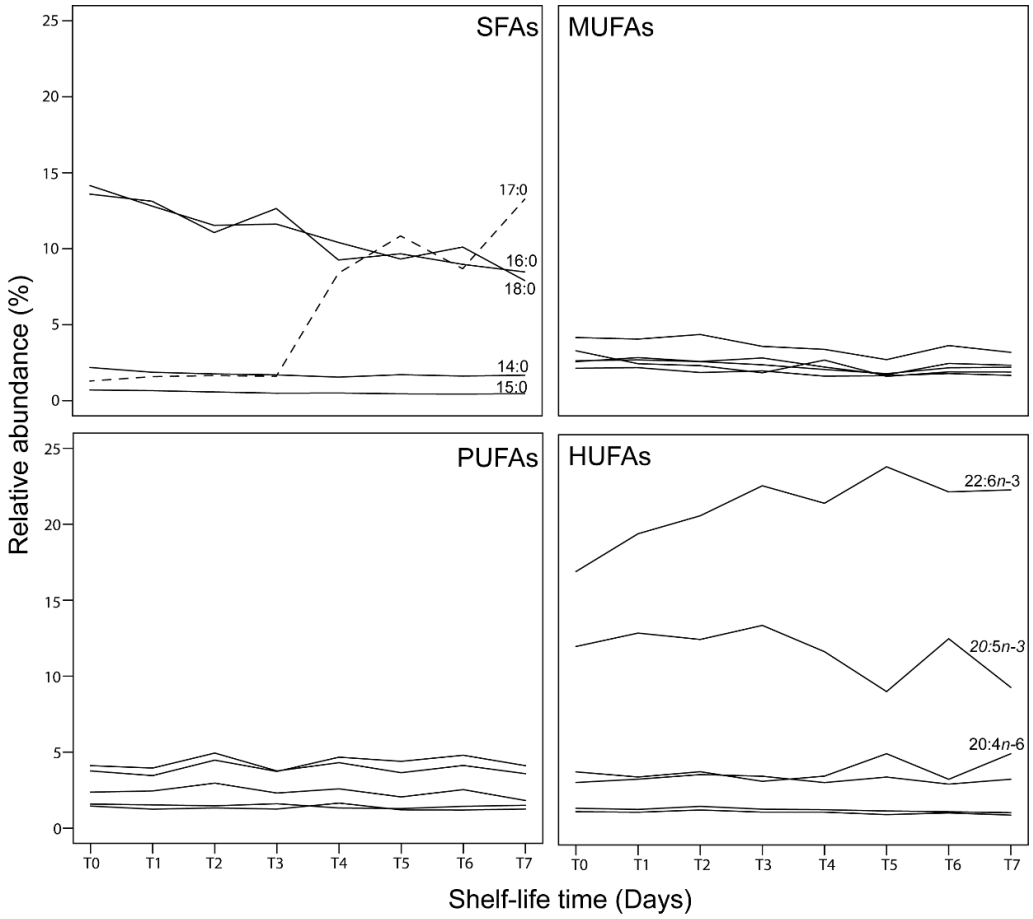

Figure 15. Relative abundances (%) of individual fatty acids within each class in the adductor muscle of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and highlyunsaturated fatty acids (HUFA).

The MANOVA revealed the existence of significant differences among the FA profiles of the AM of *C. edule* during shelf-life (F = 1.47, p = 0.027). Considering the similarity of patterns displayed by some FA identified on each class along the study period (Figure 15), the FA varying in a similar way were grouped together to further determine any significant differences (this procedure allows to minimize type II errors when performing multiple testing on the same dataset). Three groups were created for SFA (14:0 +15:0, 17:0 and 16:0 + 18:0) and HUFA (20:5*n*-3, 22:6*n*-3 and remaining HUFA). For MUFA and PUFA all FA within these classes were analysed together (one group for each class). For PUFA and the group of remaining HUFA no significant differences were recorded along the shelf-life of cockles, all other groups displayed significant differences (Table 5). The highest abundance of 17:0 and the lowest abundances of 16:0 and 18:0 were usually recorded from T4 to T7 (Figure 14), with their relative contribution to the total pool of FA being significantly different from that recorded from T0 to T3 (Figure 16; Tukey's HSD, $p < 0.05$). The relative abundance of DHA increased along shelf-life, with significant differences being recorded between T0 and T3, T5, T6 and T7 (Figure 16; Tukey's HSD, $p < 0.05$).

Table 5. Multivariate analysis of variance (MANOVA) among groups of fatty acids (FA) of the adductor muscle of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC.

FA Group	df	sumsq	meansq	*F*	*p*.value
14:0 + 15:0	7	0	0	2.75	0.024
17:0	7	0.085	0.012	10.70	0
16:0 + 18:0	7	0.014	0.002	5.55	0
MUFA	7	0	0	4.65	0.001
PUFA	7	0	0	1.66	0.154
20:5*n*3	7	0.009	0.001	2.48	0.037
22:6*n*3	7	0.016	0.002	3.89	0.004
Remaining HUFA	7	0	0	0.49	0.832

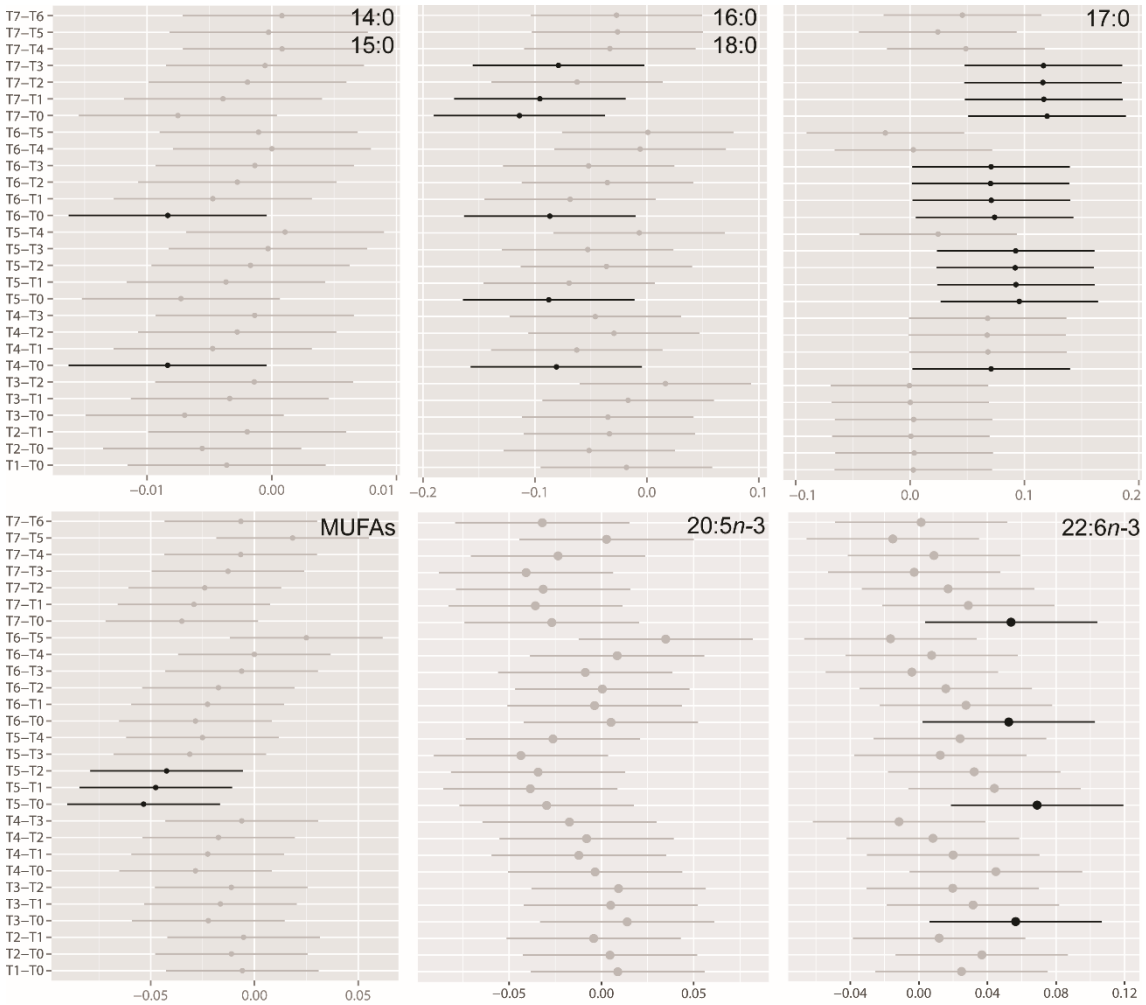

Figure 16. Significant differences (ANOVA; Tukey plot) among fatty acids 14:0+15:0, 16:0+18:0, 17:0, monounsaturated fatty acids (MUFA), 20:5*n*-3 and 22:6*n*-3 present in the adductor muscle of of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC. Significant differences ($p < 0.05$) among shelf-life times are highlighted with black lines.

The first two discriminant functions of the LDA explained 82.9% of the variation displayed by the FA profiles of the AM of cockles during shelf-life (LDA 1: 67.2 and LDA 2: 15.7%) (Figure 17). Results revealed an overall high accuracy to correctly predict the time elapsed from harvesting for each sample based upon the FA profile displayed by the AM. The overall cross-validated classification rate was 67.5% (Table 6). Samples from T3 and T7 exhibited the highest percentage of correct classifications (100%), which correspond to the upper limit of both groups defined in the correlation plot. Two replicates from T0 and T2 were misclassified, which led to an overall 80% of correct classifications. Most misclassifications were associated with specimens at T4, with no correct classifications.

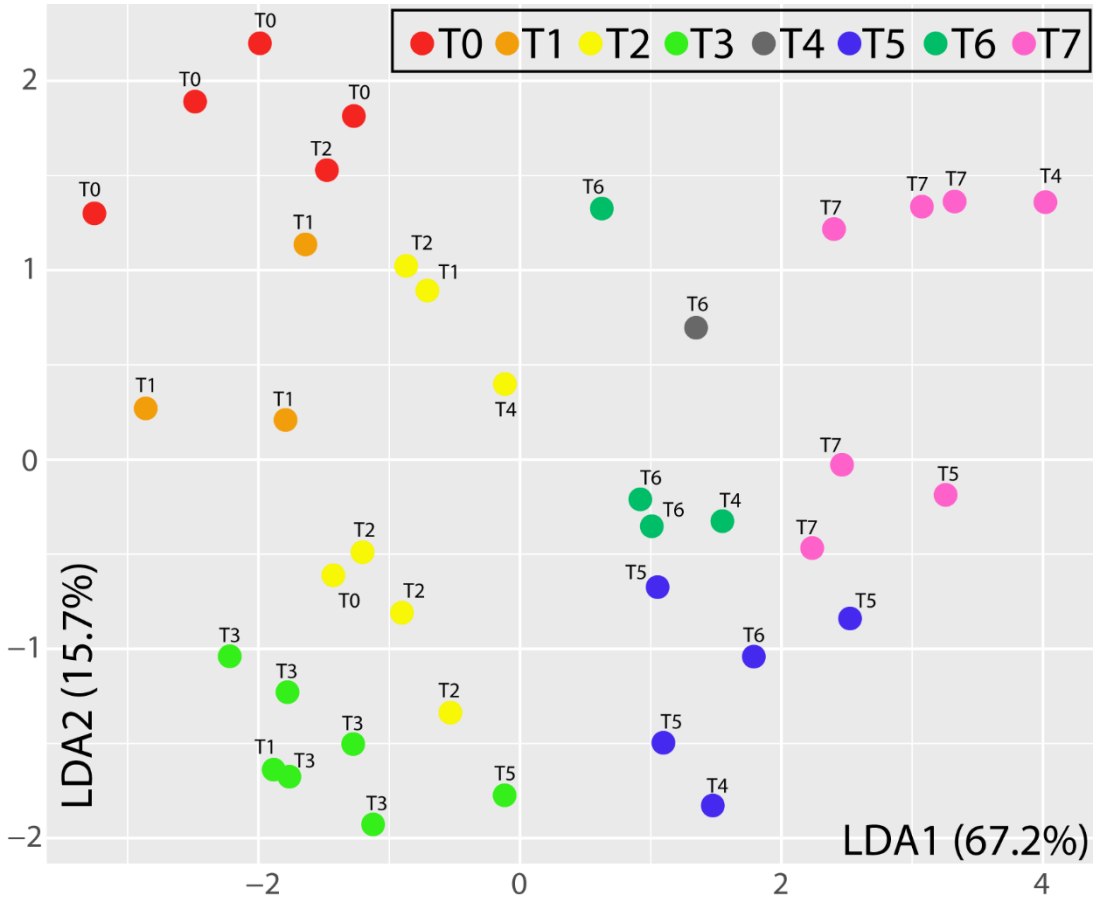

Figure 17. Linear discriminant analysis (LDA) of live common cockles *Cerastoderma edule* based on the fatty acid profiles of their adductor muscle along their shelf-life. The caption of each data point represents its true shelf-life time (expressed in days post-harvest), while colours represent the shelf-life time predicted by LDA.

Table 6. Classification success of a linear discriminant analysis (LDA) based on fatty acid profile of the adductor muscle of *Cerastoderma edule* during its shelf-life at 4 ºC.

	% Shelf-life time								Total per Shelf-life time	% correct (Shelf-life time)
	T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**		
Original Shelf-life time										
T0	80	0	20	0	0	0	0	0	5	80
T1	0	60	20	20	0	0	0	0	5	60
T2	20	0	80	0	0	0	0	0	5	80
T3	0	0	0	100	0	0	0	0	5	100
T4	20	0	20	0	0	20	20	20	5	0
T5	0	0	0	20	0	60	0	20	5	60
T6	0	0	0	0	20	20	60	0	5	60
T7	0	0	0	0	0	0	0	100	5	100
Average classification success										**67.5**

The correlation plot (Figure 18) suggested the existence of two groups: T0, T1, T2 and T3 *vs.* T4, T5, T6 and T7. The FA 17:0 was the one that mostly contributed for the differences recorded between these two groups.

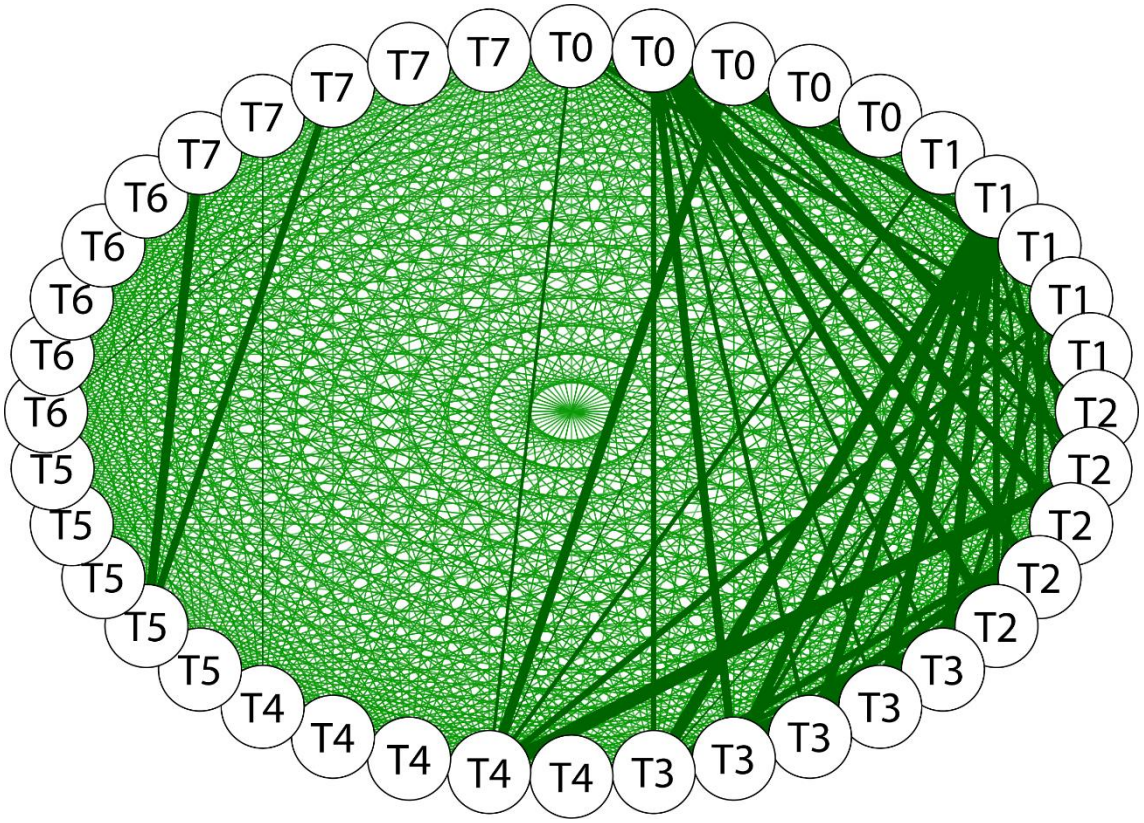

Figure 18. Correlations between shelf-life time (in days post-harvest) and fatty acids 14:0+15:0, 16:0+18:0, 17:0, monounsaturated fatty acids (MUFA), 20:5*n*-3, and 22:6*n*-3 present in the adductor muscle of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC.

2.3.4. Discussion

The FA profile of the AM of bivalves has been successfully used to trace the geographic origin of bivalves originating from locations hundreds of km apart (> 800 km) (Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen, 2010), as well as from adjacent areas in the same estuarine ecosystem (separated by less than 10 km) (Ricardo et al., 2015a). However, it remained unknown whether the FA signature of the AM of live bivalves would significantly shift during their shelf-life and consequently impair the use of this approach for tracing their geographic origin. The present study shows, for the first time, that FA profile of the AM of live cockles remains stable for most part of their shelf-life and consequently can still be use for the traceability of geographic origin of traded specimens.

The FA profile displayed by the AM of *C. edule* recorded immediately after their harvest (T0; Figure 14; Table S5 on appendix D) revealed that HUFA were predominant, followed by PUFA, SFA and MUFA. In general, the data reported in the present study for freshly harvested cockles is in line with that already published by Ricardo et al. (2015a) for specimens collected in the same arae, as well as for other bivalves, namely the fan mussel *Pinna nobilis* (Najdek, Blažina, Ezgeta-Balić, & Peharda, 2013), *P. maximus* (Grahl-Nielsen, Jacobsen, Christophersen, & Magnesen, 2010), black mussel *Mytilus galloprovincialis*, bearded horse mussel *Modiolus barbatus* and Noah's ark shell *Arca noae* (Ezgeta-Balić, Najdek, Peharda, & Blažina, 2012). In all these studies the dominant HUFA were 22:6*n*-3 and 20:5*n*-3, followed by 16:0 and 18:0 as the most abundant SFA. The odd numbered SFA 17:0 was only significant beyond T4, with the levels of this FA being the main driver for the differences recorded along the shelf-life of *C. edule* (Figure 16). The SFA 16:0 and 18:0, as well as the HUFA 22:6*n*-3, are the main FA responsible for the peculiar taste, texture and odor displayed by seafood, while the presence of the FA 17:0, as recorded from T4 to T7, is due to the presence of aerobic and anaerobic bacteria associated with spoilage (Mayzaud, Chanut, & Ackman, 1989; Najdek, Debobbis, Mioković, & Ivančić., 2002; Restuccia et al., 2015).

As live bivalves are highly perishable food items, they need to be properly handled post-harvesting, with storage at 0 to 4 ºC being a well-known method to extend their shelf-life. However, during storage, various chemical (e.g. enzymatic autolysis and lipid oxidation) and microbiological changes occur that lead to a reduction of live bivalves quality and may promote rejection of these food items by consumer prior to spoilage (Fernandes, 2016). Along the shelf-life of live cockles, FA profiles of the AM showed the existence of two main groups, with a strong correlation being recorded between specimens sampled at T0 to T3 and specimens sampled from T4 to T7 (Figure 17). This shift in FA profile of the AM of live cockles during their shelf-life (when stored refrigerated at 4 ºC) had never been reported for bivalves, with previous studies focusing on processing and seasonal driven changes (Chu, Webb, & Chen, 1990; Ezgeta-Balić, Najdek, Peharda, & Blažina, 2012; Leal, Pimentel, Ricardo, Rosa, & Calado, 2015; Ojea et al.,

2004). Overall, the FA profile of the AM of live cockles is not constant along shelf-life and at the fourth day post-harvest (T4) significant shifts start to be recorded and may confound spatial discrimination, thus impairing the traceability of geographic origin. In this way, it can be advocated that the use of FA signatures in the AM of live bivalves for traceability of their geographic origin, as described by Ricardo et al. (2015a), can be reliably employed in specimens kept refrigerated until their third day post-harvest. Moreover, it is also important to highlight that employing FA analysis of the AM of live bivalves beyond the tipping point of their FA profile post-harvest (from T3 to T4) is likely to be of little use from a commercial point of view. Indeed, at the time results will be available to verify geographic origin (1 to 3 days post-sampling), live bivalves will be too close to the limit of their shelf-life and spoilage may already start to be perceptible.

CHAPTER 3. THE USE OF TRACE ELEMENTS FINGERPRINTS IN BIVALVES TRACEABILITY

3.1. TRACE ELEMENT FINGERPRINTING OF COCKLE (*CERASTODERMA EDULE*) SHELLS CAN REVEAL HARVESTING LOCATION IN ADJACENT AREAS

3.2. SPATIO-TEMPORAL VARIABILITY OF TRACE ELEMENTS FINGERPRINTS IN COCKLE (*CERASTODERMA EDULE*) SHELLS AND ITS RELEVANCE FOR TRACING GEOGRAPHIC ORIGIN

CHAPTER 3. THE USE OF TRACE ELEMENT FINGERPRINTS IN BIVALVES TRACEABILITY

3.1. Trace element fingerprinting of cockle (*Cerastoderma edule*) shells can reveal harvesting location in adjacent areas

The material & methods, results and discussion presented in this section were integrally published as follow:

Fernando Ricardo, Luciana Génio, Miguel Costa Leal, Rui Albuquerque, Henrique Queiroga, Rui Rosa & Ricardo Calado (2015). Trace element fingerprinting of cockle (*Cerastoderma edule*) shells can reveal harvesting location in adjacent areas. *Scientific Reports 5*, 11932.

3.1.1. Background and aim of the study

Determining seafood geographic origin is critical for controlling its quality and safeguarding the interest of consumers. Here, we use trace element fingerprinting (TEF) of bivalve shells to discriminate the geographic origin of specimens. Barium (Ba), manganese (Mn), magnesium (Mg), strontium (Sr) and lead (Pb) were quantified in cockle shells (*Cerastoderma edule*) captured with two fishing methods (by hand and by hand-raking) and from five adjacent fishing locations within an estuarine system (Ria de Aveiro, Portugal). The present study aimed to validate TEF of shells from fresh bivalves as a proxy to discriminate the origin of specimens collected from adjacent areas of the same estuarine system. It is important to highlight, that unlike previous studies on TEF that use laser ablation of a small part of the larval or early juvenile shells of bivalves (Becker, Fodrie, McMillan, & Levin, 2004; Zacherl, 2005), the present study uses the whole shell of adult bivalves. The rationale for using this approach was to somehow minimize the temporal variability of TEF in the shells of adult specimens. We used cockle (*C. edule*) as a model species due to its economic importance as a fishery resource (Pereira, Maia, & Gaspar, 2013), with the coastal lagoon Ria de Aveiro (Portugal) being selected as the collection site due to its diverse tidal system and important role in Portuguese bivalve fisheries (Pereira, Maia, & Gaspar, 2013). Once cockles are usually fished by hand or hand-raking, this study also aimed to test if the use of metal rakes could induce some type of metal contamination and be a source of bias for TEF. The following hypotheses were tested i) TEF of *C. edule* shell does not differ with fishing method (i.e. hand-raking vs.by hand), and ii) TEF of *C. edule* shell is similar among different locations within the same coastal lagoon.

3.1.2. Material and methods

3.1.2.1. Study area and sample collection

C. edule with a shell length > 25 mm (i.e. commercial size) (likely displaying an age of 3+ years; the species lifespan may be up to 6 years (Malham, Hutchinson, & Longshaw, 2012) were collected during June 2013 in five different locations of Ria de Aveiro distributed among Mira (M1 and M2), Espinheiro (E1 and E2) and Ílhavo (I) Channels (Figure 19). All locations play an important role on the fishery of *C. edule* in Ria de Aveiro, which usually exceeds 1000 tons per year in this region (Pereira, Maia, & Gaspar, 2013). Two fishing methods were used to collect twenty specimens of *C. edule* at M1: ten by hand-raking and ten by hand (n = 10 *2). Subsequently, ten specimens were collected by hand on the other locations: M2, E1, E2 and I (Figure 19). All samples were stored in aseptic bags kept refrigerated during sampling and brought to the laboratory and frozen at − 20 °C for later processing

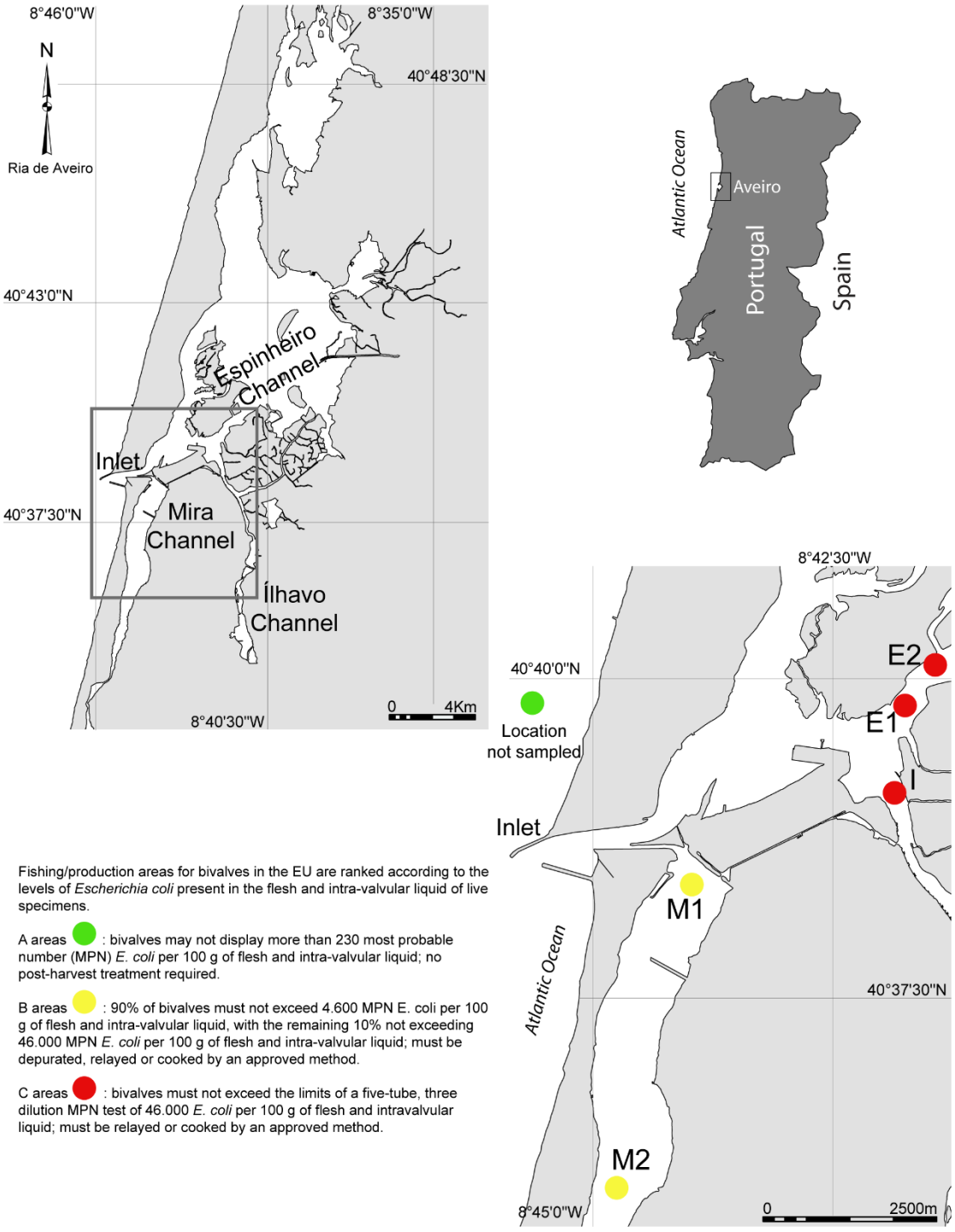

Figure 19. Sampling locations of *Cerastoderma edule* in Ria de Aveiro, Portugal: Mira Channel (M1/M1A: 40°38'26.30"N, 8°43'58.90"W and M2: 40°35'58.30"N, 8°44'47.80"W), Ílhavo Channel (I: 40°38'35.40"N, 8°41'35.40"W) and Espinheiro Channel (E1: 40°39'48.50"N, 8°41'45.03"W and E2: 40°40'2.72"N, 8°41'26.08"W). The map was created using the software ArcGIS v9.2.

3.1.2.2. Study area and sample collection

Volumetric polyethylene material and micropipettes with plastic tips were used to prepare collected shells for trace elements analysis (Özden, Erkan, & Deval, 2009). Plastic bottles, ceramic coated blades and tweezers kept in 2–5% solution of DECON 90 over 2 h were washed with running water, immersed in 10% of HNO_3 for 24 h, washed with Milli - Q (Millipore) water and dried in a laminar flow hood. The preparation for ICP-MS analysis was performed in a class 100 (ISO class 5) clean room. The valves were separated and the organic tissues were removed using ceramic coated blades and tweezers. The right valve was transferred to a previously acid-washed plastic bottle and the left valve discarded.

Samples were soaked in 20 mL high-purity H_2O_2 (30% w/v) (AnalaR NORMAPUR, VWR Scientific Products) overnight (14–16 h) to remove organic matter from the shell including the periostracum. After organic matter removal, the valve was rinsed in Milli – Q (Millipore) water three times. Digestion of entire valves was performed with addition of 20 mL of high-purity concentrated (70% w/v) HNO_3 (Trace metals; Sigma-Aldrich). To avoid having Ca masking the concentrations of the remaining elements (Elsdon & Gillanders, 2003; Ravera, Cenci, Beone, Dantas, & Lodigiani, 2003), the resulting solution was diluted with Milli – Q (Millipore) water to a final acid concentration of 2% HNO_3.

3.1.2.3. ICP-MS analysis

Samples were analysed for total aluminium (Al), barium (Ba), calcium (Ca), cadmium (Cd), copper (Cu), magnesium (Mg), manganese (Mn), lead (Pb), strontium (Sr) and zinc (Zn) by an accredited laboratory at the University of Aveiro (Portugal). The concentrations of ^{27}Al, ^{137}Ba, ^{111}Cd, ^{65}Cu, ^{55}Mn and ^{66}Zn were determined through inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS X-Series equipped with a auto sampler CETAX ASX-510, Peltier Nebulizing Camera Burgener nebulizer, nickel cones and the CeO+ /Ce+ ratio was optimized at < 2%. The concentrations of ^{48}Ca, ^{24}Mg and ^{88}Sr were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) on a ICP-OES Jobin Yvon Activa M equipped with auto sampler JY-AS500 and Burgener Mira Mist nebulizer.

3.1.2.4. Statistical analysis

Concentrations of trace elements of the shells were standardized to Ca and all data analyses were carried out on the element ratios (X: ^{48}Ca) (Becker, Fodrie, McMillan, & Levin, 2004; Strasser, Mullineaux, & Thorrold, 2008; Swearer, Forrester, Steele, Brooks, & Lea, 2003). To assess if fishing method significantly affected TEF, a resemblance matrix based on the normalized Euclidean distance was calculated (Clarke, 1993) for a one-way analysis of similarity (ANOSIM) (Clarke & Gorley, 2006), which calculates a global R statistic that assesses the

differences in variability between groups when compared to within groups and checks for the significance of R using permutation tests (Clarke & Gorley, 2006). Differences among fishing locations for each elemental ratio were assessed using a one-way analysis of variance (ANOVA), and Tukey's HSD pairwise comparisons when significant differences were observed ($p < 0.05$). Similarity percentages (SIMPER) were calculated to quantify the contribution of each trace element to the dissimilarities recorded among locations. Only trace elements that cumulatively contributed up to 80% of the dissimilarities recorded were selected (Anderson & Willis, 2003). A Canonical Analysis of Principal Coordinates (CAP) (Anderson & Willis, 2003) was performed to test if TEF could be used to predict the fishing location of collected specimens. CAP is a constrained ordination tool that discriminates locations defined a priori and determines the level of misclassification among sampling locations. Appropriate axis (m) was applied by maximizing the leave-one-out allocation success ($m = 5$) (Anderson, Clarke, & Gorley, 2008). This approach tests how well locations were discriminated using CAP. To quantify the effect of each trace element to potential differences recorded among locations, Spearman correlation were calculated for all trace elements and the CAP axes. Only the trace elements with a correlation coefficient $|r| > 0.30$ were considered. ANOVAs were performed using GraphPad Prism 6 (GraphPad Software. Inc., San Diego, CA, USA), and multivariate analyses were performed using PRIMER v6 with the add-on PERMANOVA + .

3.1.3. Results

Five trace elements (^{137}Ba, ^{24}Mg, ^{55}Mn, ^{207}Pb and ^{88}Sr) were detected in *C. edule* shells from Ria de Aveiro, with Mg and Sr denoting the highest ratios to Ca (Figure 20). While no differences between specimens collected by hand or by hand-raking were detected (ANOSIM, $p = 0.268$, $R = 0.025$), significant differences among locations were observed for each trace element ratio (Figure 20; one-way ANOVA, $p < 0.05$ for all trace elements; Table 7, summarizes ANOSIM results). The ratios of Mn and Ba were significantly higher at location M2 ($p = 0.0001$ and 0.0001, respectively). In contrast, the Mg ratio was lowest for *C. edule* shells from M2 ($p = 0.0004$). The Pb ratio was only significantly higher ($p = 0.0001$) at location I, whereas the Sr ratio was also higher at this location but only significantly different from shells collected at E2.

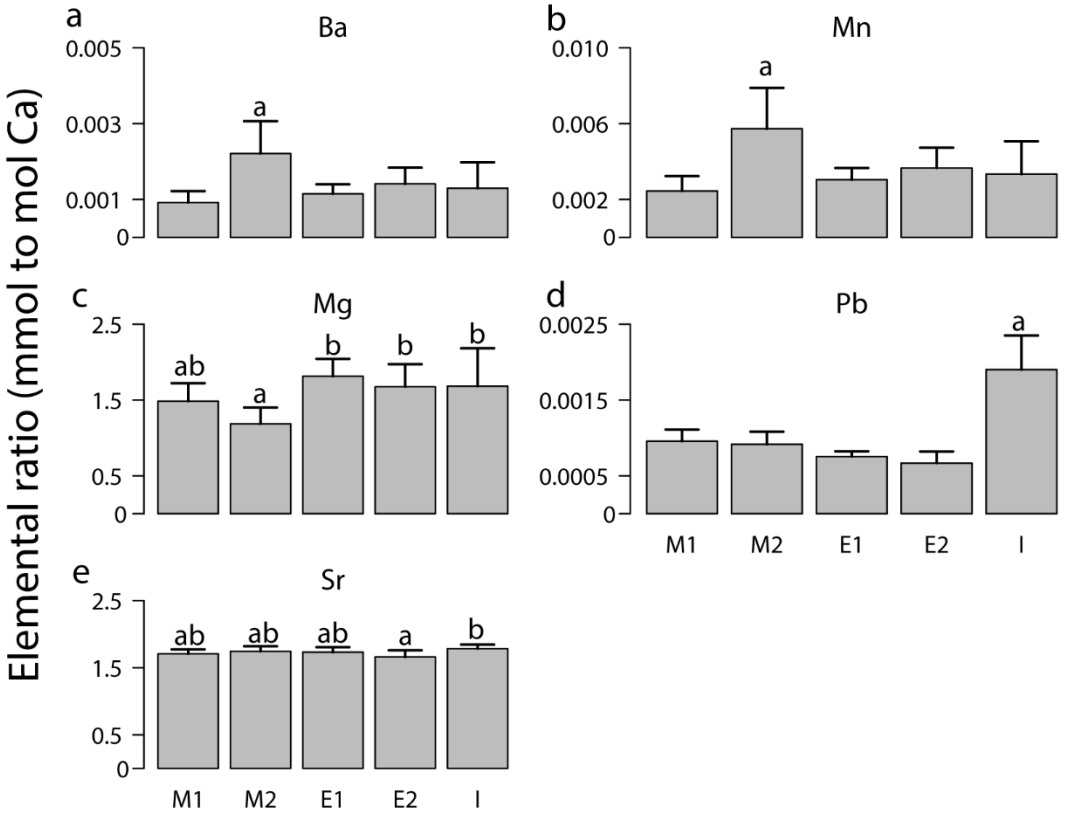

Figure 20. Ratios of trace elements to Calcium (Ca) concentrations (mmol to mol) (average ± SD; n = 10) of *Cerastoderma edule* shells from five locations within Mira (M1 and M2), Espinheiro (E1 and E2) and Ílhavo (I) Channels in Ria de Aveiro (Portugal). Significant differences ($p < 0.05$) among different locations are noted with different letters.

Table 7. Similarity (ANOSIM) between trace elements fingerprinting of *Cerastoderma edule* shell from five locations within Mira (M1 and M2), Espinheiro (E1 and E2) and Ílhavo (I) Channels in Ria de Aveiro (Portugal).

Locations	*R*	*p*
M1 vs M2	0.503	0.002
M1 vs E1	0.238	0.025
M1 vs E2	0.202	0.028
M1 vs I	0.401	0.002
M2 vs E1	0.641	0.001
M2 vs E2	0.428	0.001
M2 vs I	0.563	0.001
E1 vs E2	0.123	0.059
E1 vs I	0.673	0.001
E2 vs I	0.643	0.001

Pairwise comparisons revealed significant differences among locations, apart from those within the Espinheiro Channel, i.e. E1 and E2 (ANOSIM, $p = 0.059$, R = 0.123). SIMPER analysis showed that the dissimilarity among locations was associated to five elemental ratios: Mg, Sr, Pb, Ba and Mn. (Table 8). Mg and Sr were always among the elements that most contributed for the variability between location M1 and locations from Espinheiro Channel (E1 and E2). Mg and Sr varied significantly between M1 and Espinheiro ($p = 0.0001$ and 0.021,

respectively) and SIMPER revealed that these elements explained more than 55% of the differences recorded between these locations (Table 8). Specimens from location I were significantly different from other areas due to their concentrations of Pb (Figure 20). SIMPER analysis revealed that Pb alone accounted for 28 to 53% of all differences recorded between location I and all other locations (Table 8). Ba and Mn together contributed for more than 43% of the differences recorded among specimens collected in M2 and other locations.

Table 8. Similarity percentage analysis (SIMPER) identifying the elements contributing to the differences recorded in the shell of *Cerastoderma edule* from five locations within Mira (M1 and M2), Espinheiro (E1 and E2) and Ílhavo (I) channels in Ria de Aveiro, Portugal (Ind – individual; Cum – cumulative).

M1 vs M2			**M2 vs E2**		
Element	**Ind (%)**	**Cum (%)**	**Element**	**Ind (%)**	**Cum (%)**
Mn	38.52	38.52	Mn	25.26	25.26
Ba	38.47	76.99	Ba	24.96	50.22
Sr	11.31	88.31	Sr	24.18	74.40
			Mg	21.73	96.13
M1 vs E1			**M2 vs I**		
Element	**Ind (%)**	**Cum (%)**	**Element**	**Ind (%)**	**Cum (%)**
Mg	38.32	38.32	Pb	28.01	28.01
Sr	34.08	72.40	Mn	22.23	50.25
Mn	10.16	82.56	Ba	21.66	71.91
			Mg	20.40	92.31
M1 vs E2			**E1 vs E2**		
Element	**Ind (%)**	**Cum (%)**	**Element**	**Ind (%)**	**Cum (%)**
Sr	35.57	35.57	Sr	52.55	52.55
Mg	20.80	56.37	Mg	21.40	73.94
Ba	17.35	73.72	Ba	11.91	85.85
Mn	16.86	90.58			
M1 vs I			**E1 vs I**		
Element	**Ind (%)**	**Cum (%)**	**Element**	**Ind (%)**	**Cum (%)**
Pb	41.50	41.50	Pb	53.86	53.86
Mg	19.69	61.19	Mg	17.12	70.99
Sr	16.24	77.42	Sr	12.62	83.60
Ba	11.41	88.84			
M2 vs E1			**E2 vs I**		
Element	**Ind (%)**	**Cum (%)**	**Element**	**Ind (%)**	**Cum (%)**
Ba	29.75	29.75	Pb	46.98	46.98
Mn	29.56	59.31	Sr	24.10	71.08
Mg	27.61	86.93	Mg	13.73	84.81

TEF differences among locations were strong enough to accurately assign collected specimens to their fishing location. The leave-one-out procedure resulted in an average CAP classification of 92% (Table 9), i.e. 92% of the specimens were correctly assigned to their origin. Locations M1, M2 and I had the highest percentage of correct classification (100%), whereas two replicates from E1 and E2 were misclassified, which led to 80% correct classifications. Vector overlay of Spearman correlations of TEF with CAP axes are shown in Figure 21. Vectors of Ba and Mn ratios were positively correlated with samples from location M2, Mg ratio with areas E1 and E2, and Pb and Sr ratios associated with samples from location I (Figure 21). *C. edule* from area M1 were not associated with a particular trace element.

Table 9. Classification success of cross-validation for cockle *Cerastoderma edule* based on trace elemental composition in the shell from five locations within Mira (M1 and M2), Espinheiro (E1 and E2) and Ílhavo (I) Channels in Ria de Aveiro, Portugal.

	Predicted Locations					**Total per location**	**% correct (location)**
	M1	**M2**	**E1**	**E2**	**I**		
Original Area							
M1	10					10	100
M2		10				10	100
E1			8	2		10	80
E2			2	8		10	80
I					10	10	100
Average classification success							92

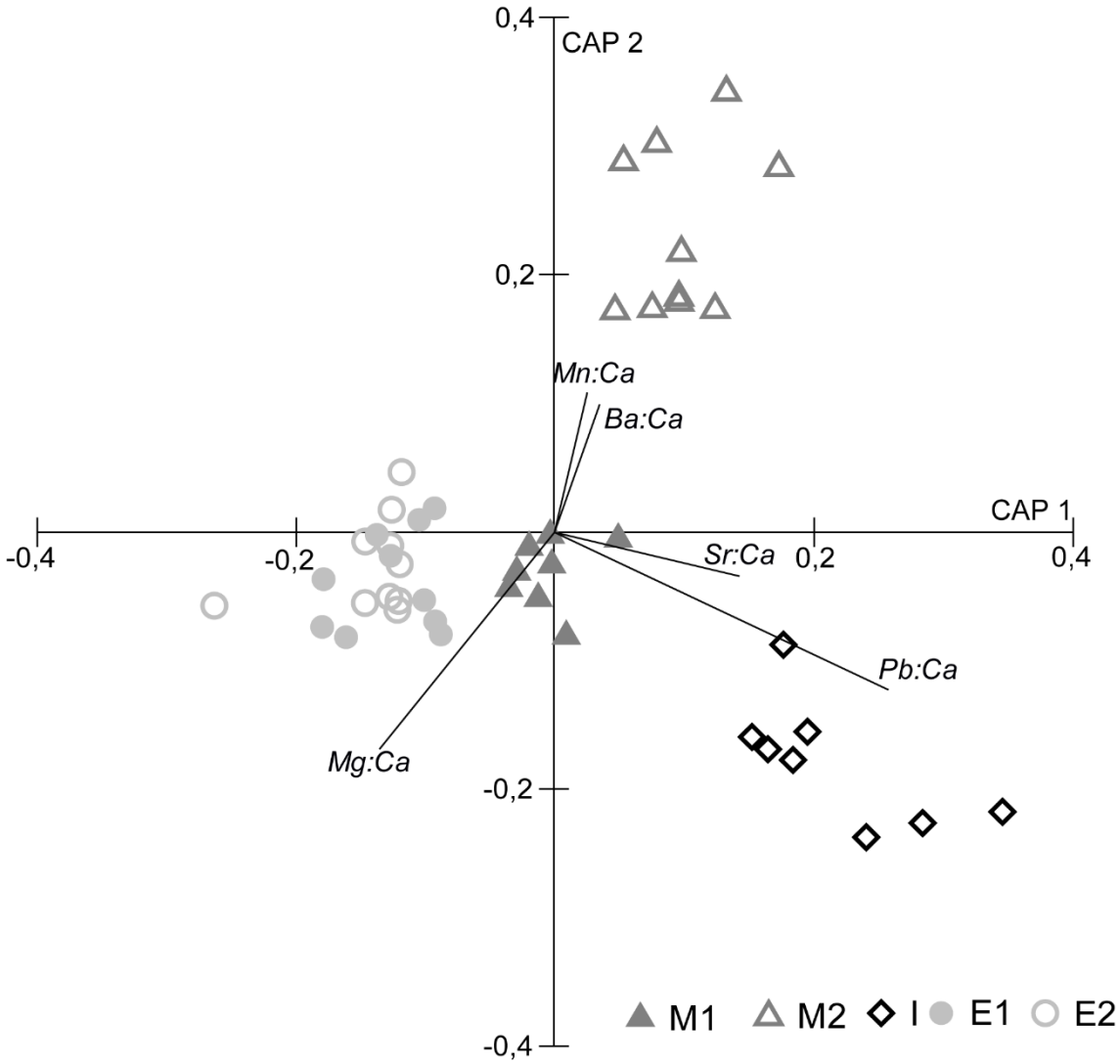

Figure 21. Canonical analysis of principal coordinates (CAP) based on Euclidean distances matrices of normalized elemental ratios, with axes drawn to maximize discrimination among assemblage types. Vector overlay Spearman correlations of trace elements composition with canonical axes are shown if |r| > 0.30.

3.2.4. Discussion

In general, adult bivalves display a reduced locomotor ability, being their aragonitic shells potential biogenic archives of marine ecosystems environmental fingerprints (Lavaud, Thébault, Lorrain, van der Geest, & Chauvaud, 2013). This feature prompted the use of trace elements of bivalve shells to assess their geographic origin. TEF has been successfully used to geographically distinguish populations of blue mussel *M. edulis* (Sorte, Etter, Spackman, Boyle, & Hannigan, 2013), black mussel *M. galloprovincialis* and California sea mussel *M. californianus* (Becker, Fodrie, McMillan, & Levin, 2004; Carson et al., 2013), soft shell clam *Mya arenaria* (Strasser, Mullineaux, & Thorrold, 2008) and Olympia oyster *Ostrea lurida* (Carson, 2010). This geochemical tool also allowed to distinguish juveniles of green-lipped mussel (*Perna canaliculus*) ~13 km apart (Dunphy, Millet, & Jeffs, 2011), and to record differences in scallop shells (*Argopecten irradians*) within a small bay (~10 km^2) (Broadaway & Hannigan, 2012). The

present study shows, for the first time, that TEF of bivalve shells can be used to assign the origin location of bivalves with a resolution <1 km.

While Mg and Sr ratios were relatively higher than Ba and Mn (Figure 20), the latter ratios were among the most important to differentiate locations (Table 8). The presence of Ba and Mn with elevated concentration, as observed in M2, have been already reported for *Isognomon ephippium* (Lazareth, Putten, André, & Dehairs, 2003), *Mercenaria mercenaria*, *Spisula solidissima* (Stecher, Krantz, Lord, Luther, & Bock, 1996) and *M. edulis* (Putten, Dehairs, Keppens, & Baeyens, 2000). Such high concentration in Ba and Mn are usually associated with freshwater inputs and nutrient runoff to estuarine systems, which ultimately causes phytoplankton blooms, particularly diatoms (Gillikin et al., 2006; Putten, Dehairs, Keppens, & Baeyens, 2000; Stecher, Krantz, Lord, Luther, & Bock, 1996; Thébault et al., 2009). It is possible that the environmental conditions at M2, which is located more upstream and has stronger riverine input, causes diatom blooms more often than the conditions observed at others locations such as M1, which is located near the inlet (Cerejo & Dias, 2007). Ba and Mn end up in bivalves' tissue and shell as a consequence of the ingestion of Ba and Mn-rich particles associated with such diatom blooms. Heavy metals are also incorporated in calcite and aragonite shells of bivalves (Bourgoin, 1990; Pitts & Wallace, 1994). The high levels of Pb in the shells from location I are likely associated with anthropogenic impacts, particularly acute pollution from boats using leaded gasoline. Note that location I is relatively close to the commercial harbour of Aveiro.

Cockle shells from each location displayed a different TEF, with the exception of stations E1 and E2 that showed no statistical differences between each other (Table 7). Nevertheless, CAP results showed a success of 80–100% to identify the origin of cockles collected from Ria de Aveiro (Table 9). The important to highlight that the misclassifications were solely associated with locations E1 and E2. It is the potential of TEF for geographical traceability purposes as we were able to identify the origin of cockles using this statistical tool (CAP) in, at least, 80% of the cases. Nevertheless, the average 92% correct classification is still higher than results by Sorte, Etter, Spackman, Boyle, & Hannigan (2013) for the blue mussel *M. edulis* in the Gulf of Maine (68%) and by Becker, Fodrie, McMillan, & Levin (2004) for the congeners mussels *M. californianus* and *M. galloprovinciallis* in Southern California, USA (56%). The latter and other studies (Becker, Fodrie, McMillan, & Levin, 2004; Carson, 2010; Carson et al., 2013; Dunphy, Millet, & Jeffs, 2011; Sorte, Etter, Spackman, Boyle, & Hannigan, 2013) have also shown that Ba, Mn, Mg, Pb and Sr play an important role discriminating specimens among areas, as observed here through the magnitude of the vectors of the standardized discriminant functions (Figure 21).

The chemical nature of the trace elements deposited over time in bivalves is determined by metabolic efficiency and environmental conditions (Yan, Chen, & Xiao, 2014). As this study was conducted in Ria de Aveiro, which is a highly dynamic tidal-system with notable spatial variability in environmental conditions (Dias, Lopes, & Dekeyser, 1999), it is likely that different

fingerprints are associated with contrasting environmental conditions recorded at each channel (Figure 19). The spatial variability here recorded for TEF of cockle shells thus stresses the potential of this method to validate screening for fraudulent use of origin certification. However, temporal variability in environmental conditions may also change TEF and interfere with this traceability tool. Indeed, it has already been shown that seasonal and annual variation may change the TEF of bivalves and other biogenic carbonate structures such as fish otoliths (Becker, Fodrie, McMillan, & Levin, 2004; Carson et al., 2013; D'Avignon & Rose, 2013). In opposite, Carré, Bentaleb, Bruguier, et al. (2006) showed that environmental changes have minor influence on Sr, Ba, Mg and Mn concentration in shell aragonite of the marine bivalve species *Mesodesma donacium* and *Chione subrugosa*. This study aimed to validate a tool for origin certification of bivalves and not to study the temporal variability of TEF. Consequently, we used cockles with similar size and, therefore, similar age, in order to minimize any bias associated with potential differences in the age of selected specimens. The analyses performed in this study used the whole shell and, consequently, averaged the present and the past elemental fingerprints of cockles. While this approach may have the TEF over multiple years, notable differences among sites were still recorded (Table 7), which emphasizes the robustness of this method for geographical traceability purposes. However, if legal authorities aiming to fight the fraudulent mislabelling of origin location want to minimize this potential temporal bias associated with the analysis of the whole shell, they may rather monitor the elemental fingerprint of the outer margins of bivalve shells from each fishing location or, those more prone to fraud, as these will reflect the most recent elemental fingerprints from the location where they were collected. By comparing the fingerprint of the investigated shells with monitoring data and/or samples from the different sites in the same season, legal authorities may minimize the effect of temporal variability and, ultimately, use of this tool to expose fraudulent situations.

Although TEF fails to detect differences associated with fishing method, this information would be potentially relevant for legal authorities to manage bivalve trade, from fishing to the end consumer, as fishermen using hand-raking usually collect larger volumes of bivalves. The effect of environmental conditions on the TEF of bivalves occurs within a relatively long time frame, i.e. within weeks or months (Klumpp & Burdon-Jones, 1982), which likely explains the lack of differences associated with fishing methods. The effect of fishing method on TEF, if any, would probably occur within a very small time frame as fishing duration usually takes less than one hour.

Most traceability tools have been focused on issues associated with species mislabelling (Espiñeira, Gonzalez-Lavin, Vieites, & Santaclara, 2009; Garcia-Vazquez et al., 2010; Herrero, Lago, Vieites, & Espiñeira et al., 2012) or with identification of geographical origin of specimens separated by distances higher than 20 km (Becker, Fodrie, McMillan, & Levin., 2004; Sorte, Etter, Spackman, Boyle, & Hannigan, 2013). However this study shows, for the first time, that TEF can

be a fast, reliable and accurate method that may be used for origin certification of bivalves collected from locations less than 1 km apart. While this is probably associated with the high environmental variability observed within Ria de Aveiro, it is still unknown if TEF is a reliable tool to accurately identify the origin of bivalves collected from different ecosystems with similarly high variability. Follow-up studies are already being developed to clarify if TEF can be used to discriminate between bivalve shells from specimens originating from distinct ecosystems (from tens to hundreds of km apart). An additional benefit of TEF is that there is no post-harvesting shift and/or degradation associated with bacterial action as recorded for biochemical and molecular methods. The present approach may also play a relevant role on the conservation and management of cockle populations being exploited, namely in the fight against illegal/unreported fishing.

CHAPTER 3. THE USE OF TRACE ELEMENT FINGERPRINTS IN BIVALVES TRACEABILITY

3.2. Spatio-temporal variability of trace elements fingerprints in cockle (*Cerastoderma edule*) shells and its relevance for tracing geographic origin

The material & methods, results and discussion presented in this section were integrally published as follow:

Fernando Ricardo, Tânia Pimentel, Luciana Génio & Ricardo Calado (2017). Spatio-temporal variability of trace elements fingerprints in cockle (*Cerastoderma edule*) shells and its relevance for tracing geographic origin. *Scientific Reports 7*, 3475.

3.2.1. Background and aim of the study

Understanding spatio-temporal variability of trace elements fingerprints (TEF) in bivalve shells is paramount to determine the discrimination power of this analytical approach and secure traceability along supply chains. The aim of this study was to evaluate the spatio-temporal variability of trace elements fingerprints (TEF) of cockle (*Cerastoderma edule*) shells and use them to assess their geographic origin. Spatial variability of TEF was determined in specimens captured in eight different ecosystems along the Portuguese coast (Ria de Aveiro, Óbidos lagoon, Tagus estuary, Albufeira lagoon, Sado estuary, Mira estuary, Ria de Alvor and Ria Formosa). The present study also aimed to determine the temporal stability of TEF in cockle shells between two consecutive years (2013 and 2014) in areas within the same ecosystem but displaying different classifications (according to European regulation (EC) No 1379/2013 (EC, 2013) for the capture/production of bivalves.

3.2.2.Material and methods

3.2.2.1. Study area and sample collection

Cockles (*C. edule*) were collected during low tide over June-July (Summer) 2014 on eight estuarine ecosystems along the Portuguese coast (Figure 22): Ria de Aveiro (RAv) (Figure 22a), Óbidos Lagoon (OL) (Figure 22b), Tagus Estuary (TE), Albufeira Lagoon (AL), Sado Estuary (SE) (Figure 22c), Mira Estuary (ME) (Figure 22d), Ria de Alvor (RAl) (Figure 22e) and Ria Formosa (RF) (Figure 22f). At the sampling moment, all locations were classified as "B" or "C" (Fig. 1) according to Council Regulation 853/2004 and 854/2004 of the European Union (EU) (EC, 2004a, 2008). In RAv four different areas were sampled and ten cockles collected per area (1 ecosystems X 4 areas X 10 replicates = 40 samples) (Figure 22a). In OL, TE, AL, SE and RF two areas were sampled, with ten cockles also being collected on each one them (5 ecosystems X 2 areas X 10 replicates = 100 samples). As the bivalve species being surveyed was not abundant in ME and RAl only one area was sampled per ecosystem, with ten cockles being collected on each of them (2 ecosystem X 1 area X 10 replicates = 20 samples).

To evaluate the temporal variability of TEF in cockle shells, samples collected in the present study in RAv (Figure 22a) were compared to those of specimens collected exactly in the same locations in the previous year (July (Summer) 2013). Ten specimens were sampled on each area in the two consecutive years (1 ecosystem X 4 areas X 2 years X 10 replicates = 80 samples). It is important to highlight that only cockles with approximately 3 years old (shell length 20-25 mm, commercial size) (Seed & Brown, 1978) were sampled and that the time window for the present study (summer) matched that when the capture and trade of this bivalve species is higher and fraudulent practices are more likely to occur. All samples were collected by hand-raking, stored in aseptic food grade plastic bags and kept refrigerated during sampling. All specimens were frozen at -20 °C in the same day of collection for further analysis.

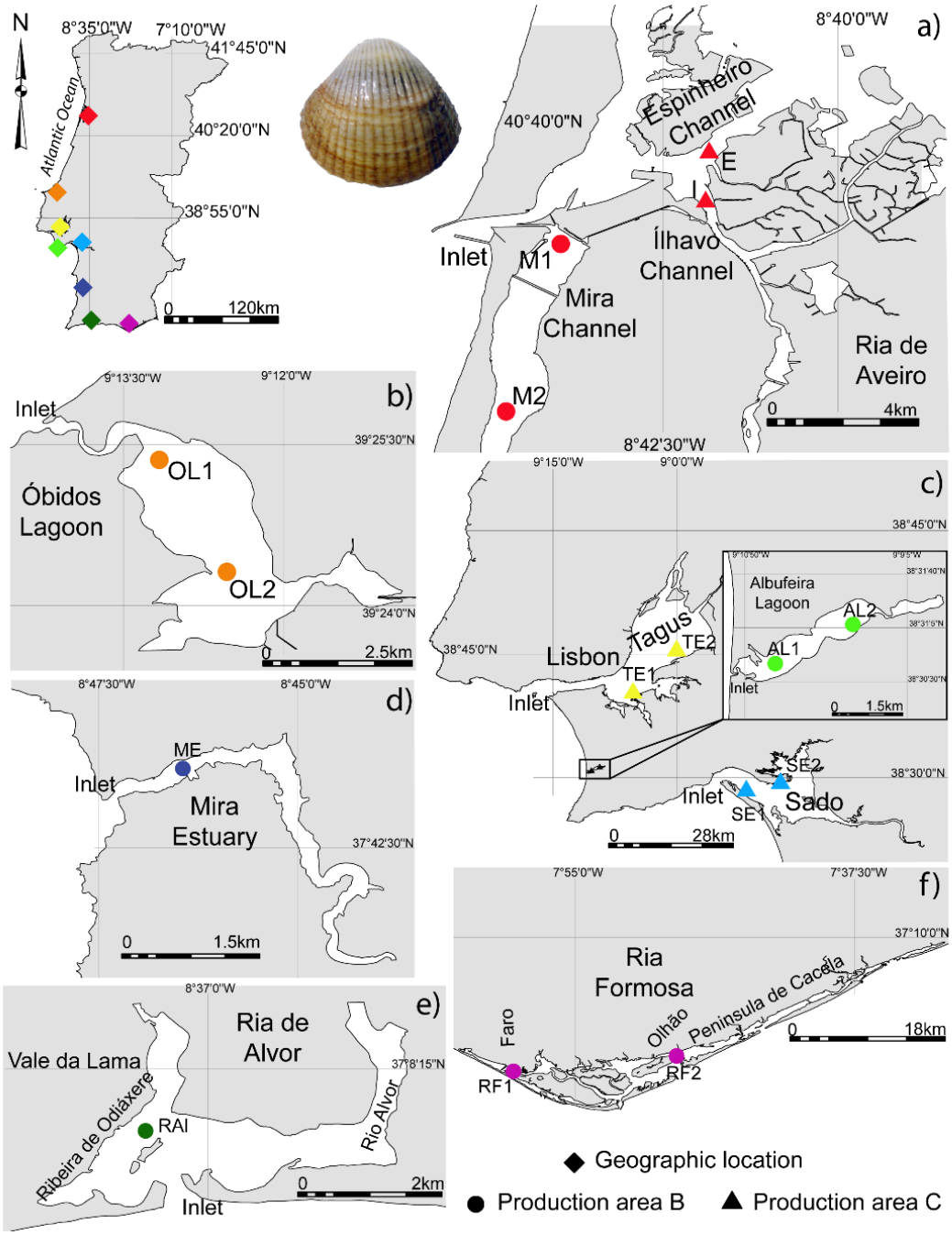

Figure 22. Sampling locations of *Cerastoderma edule* in mainland Portugal: a) Ria de Aveiro (RAv; M1:40°38'26.30"N, 8°43'58.90"W; M2: 40°35'58.30"N, 8°44'47.80"W; I: 40°38'35.40"N, 8°41'35.40"W and E: 40°39'48.50"N, 8°41'45.03"W), b) Óbidos lagoon (OL1: 39°25'20.34"N, 9°13'14.54"W and OL2: 39°24'2.01"N, 9°12'30.91"W), c) Tagus estuary (TE1: 38°39'27.44"N, 9°6'35.95"W and TE2: 38°44'5.18"N, 9°0'46.54"W), Albufeira lagoon (AL1: 38°30'36.67"N, 9°10'32.96"W and AL2: 38°31'1.33"N, 9°9'53.16"W) and Sado estuary (SE1: 38°27'46.00"N, 8°51'32.00"W and SE2: 38°29'13.25"N, 8°48'52.79"W) d) Mira estuary (ME: 37°43'30.60"N, 8°46'15.40"W), e) Ria de Alvor (RAl: 37°07'55.7"N, 8°37'27.40"W) and f) Ria Formosa (RF1: 37°00'23.20"N, 7°59'28.40"W and 37°01'24.30"N, 7°49'49.50"W). The map was created using the software ArcGIS v10.2.2.

3.2.2.2. Elemental analysis of cockle shells

Prior to elemental analysis all shells were prepared as described in detail by Ricardo et al. (2015b). The valves were separated and the organic tissues were removed using ceramic coated blades and tweezers. The whole right valve was transferred to a previously acid-washed plastic bottle and the left valve discarded. Trace elements of the whole valve were obtained by the digestion method described by Ricardo et al. (2015b). Briefly, samples were soaked in 20 mL high-purity H_2O_2 (30% w/v) (AnalaR NORMAPUR, VWR Scientific Products) overnight (14–16 h) to remove organic matter from the shell including the periostracum. After organic matter removal Digestion of entire valves was performed with addition of 20 mL of high-purity concentrated (70% w/v) HNO_3 (Trace metals; Sigma-Aldrich). To avoid having Ca masking the concentrations of the remaining elements (Elsdon et al., 2008; Ravera, Cenci, Beone, Dantas, & Lodigiani, 2003), the resulting solution was diluted with Milli – Q (Millipore) water to a final acid concentration of 2% HNO_3. Procedural blanks were prepared using the same analytical procedure and reagents of the samples. Barium (measured as ^{137}Ba) and manganese (measured as ^{55}Mn) present in *C. edule* shells were measured by inductively coupled plasma mass spectrometry (ICP-MS) on a Thermo ICP-MS X-Series equipped with an auto sampler CETAX ASX-510, Peltier Nebulizing Camera Burgener nebulizer, with nickel cones and the CeO+/Ce+ ratio being optimized at < 2%. Calcium (measured as ^{43}Ca), magnesium (measured as ^{24}Mg) and strontium (measured as ^{88}Sr) were determined through inductively coupled plasma optical emission spectrometry (ICP-OES) on a ICP-OES Jobin Yvon Activa M equipped with auto sampler JY-AS500 and Burgener Mira Mist nebulizer. The precision and accuracy of the analytical procedures were ensured by the analysis of certified reference materials (MRC's) for sediments (Table S6 on appendix E) while, the operating conditions are showed in Table S7 on appendix E.

3.2.2.3. Statistical analysis

The concentration of trace elements present in the shells of cockles was expressed as a ratio relatively to Ca (Génio, Simon, Kiel, & Cunha, 2015; Ricardo et al., 2015b), with these ratios always being calculated prior to any statistical analysis. A preliminary analysis of multivariance (MANOVA) was performed to detect significant differences ($p < 0.05$) in the TEF of shells from specimens originating from different areas within each ecosystem. As significant differences were recorded (Table S8 on appendix E), only the closest areas to the inlet from each ecosystem were selected for further analysis. The rationale supporting this decision was the assumption that the areas closer to the inlet of each ecosystem are more similar among them due to the environmental influence of ocean conditions and, consequently, these locations would likely be more challenging to discriminate TEF from cockle shells. A total of 10 replicates per ecosystem were therefore used for further statistical analysis. In order to determine whether there were any significant differences ($p < 0.05$) in the TEF of cockle shells among ecosystems an

analysis of multivariance (MANOVA) was performed. As significant differences were recorded, one-way analysis of variance (ANOVA) were performed for each elemental ratio. Whenever the ANOVA revealed the existence of significant differences ($p < 0.05$) a post hoc test (Tukey's test) was performed to identify which ecosystems differed from each other. A linear discriminant analysis (LDA) was performed to assess the reliability of using TEF displayed by cockle shells to infer their geographic origin.

A MANOVA was used to evaluate the inter-annual stability of TEF displayed by cockle shells over two consecutive years (2013 and 2014) within the four sampled areas of RAv. Due to the significant differences recorded, an ANOVA was applied (as detailed above), with post hoc tests (Tukey's test) being employed when applicable. All analyses were performed on log X+1 transformed data, in order to meet the normality and homogeneity of variance of ANOVA and the multivariate normality and homoscedasticity (Pillai Trace test) are required for MANOVA. All statistical analysis were performed using R (R Development Core Team, 2015).

3.2.3. Results

Trace element fingerprints (TEF) of *C. edule* shells differed among ecosystems, with the exception of Mira estuary (ME) and Ria de Alvor (RAl), with MANOVA analyses revealing strong significant differences when considered all trace elements together (Table 10).

Table 10. Multivariate analysis of variance (MANOVA) of trace elements fingerprints of *Cerastoderma edule* shells from eight ecosystems along the Portuguese coast: Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

Ecosystem	df	pillai	approx. F	*p*	bonf.*p*.adjust
RAv vs OL	1	0.948	67.82	0.000	0.000
RAv vs TE	1	0.946	66.20	0.000	0.000
RAv vs AL	1	0.938	56.85	0.000	0.000
RAv vs SE	1	0.943	62.61	0.000	0.000
RAv vs ME	1	0.975	147.77	0.000	0.000
RAv vs RAl	1	0.938	56.84	0.000	0.000
RAv vs RF	1	0.981	194.73	0.000	0.000
OL vs TE	1	0.948	68.35	0.000	0.000
OL vs AL	1	0.950	71.95	0.000	0.000
OL vs SE	1	0.969	117.21	0.000	0.000
OL vs ME	1	0.977	158.33	0.000	0.000
OL vs RAl	1	0.945	63.95	0.000	0.000
OL vs RF	1	0.881	27.74	0.000	0.000
TE vs AL	1	0.908	36.99	0.000	0.000

Table 10 (cont). Multivariate analysis of variance (MANOVA) of trace elements fingerprints of *Cerastoderma edule* shells from eight ecosystems along the Portuguese coast: Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

Ecosystem	df	pillai	approx. F	*p*	bonf.*p*.adjust
TE vs SE	1	0.699	8.73	0.001	0.021
TE vs ME	1	0.919	42.28	0.000	0.000
TE vs RAl	1	0.892	31.12	0.000	0.000
TE vs RF	1	0.954	77.94	0.000	0.000
AL vs SE	1	0.838	19.42	0.000	0.000
AL vs ME	1	0.936	55.20	0.000	0.000
AL vs RAl	1	0.720	9.63	0.000	0.013
AL vs RF	1	0.848	20.86	0.000	0.000
SE vs ME	1	0.935	54.02	0.000	0.000
SE vs RAl	1	0.821	17.14	0.000	0.001
SE vs RF	1	0.945	64.73	0.000	0.000
ME vs RAl	1	0.531	4.24	0.017	0.480
ME vs RF	1	0.940	59.14	0.000	0.000
RAl vs RF	1	0.891	30.68	0.000	0.000

Considering each element separately, cockles from Ria de Aveiro (RAv) and Óbidos lagoon (OL) registered the highest Mg/Ca and were not significantly different from each other ($p \geq 0.05$), but differed significantly from specimens originating from other ecosystems ($p < 0.05$) (Figure 23). Cockles from the Tagus estuary (TE) showed the highest Mn/Ca with significant differences being recorded between specimens from this ecosystem and conspecifics collected in all others ecosystems that were surveyed ($p < 0.05$) (Figure 23). Ba/Ca was higher in cockle shells from RAv, OL and Ria Formosa (RF), with significant differences being recorded when compared with that of cockles from all other ecosystems ($p < 0.05$) (Figure 23). Concerning's Sr/Ca, all specimens collected in the ecosystems surveyed in this study displayed a similar ratio for this element, although significant differences ($p < 0.05$) were still recorded between *C. edule* shells from different ecosystems (Figure 23).

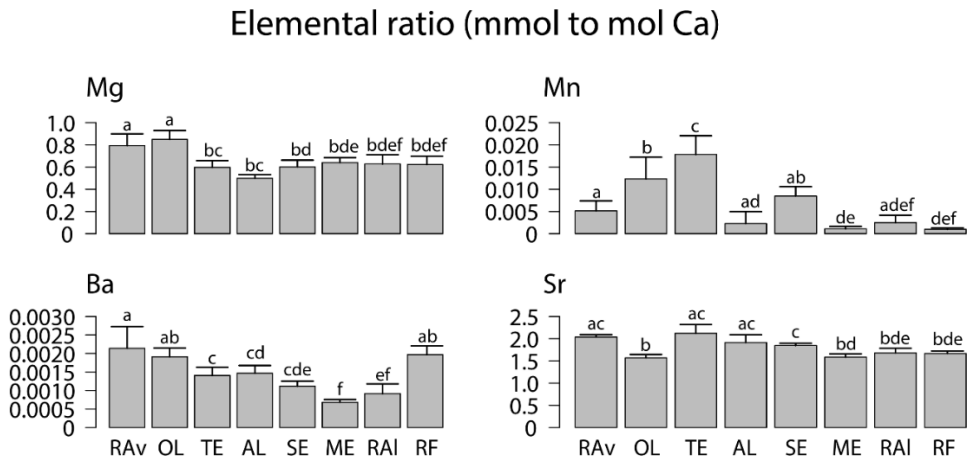

Figure 23. Ratios of trace elements to Calcium (Ca) concentrations (mmol to mol) (average ± SD; n = 10) of *Cerastoderma edule* shells from eight ecosystems along the Portuguese coast: Ria de Aveiro (RAv), Óbidos Lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF). Significant differences ($p < 0.05$) among different ecosystems are noted with different letters.

The first two discriminant functions of the linear discriminant analysis (LDA) explained 77.6% of the TEF variation in the data set (LDA 1: 47.6% and LDA 2: 30%) (Figure 24). Specimens collected in RAv, RF, OL and Sado estuary (SE) displayed the highest percentages of correct classification (100%), whereas for cockles originating from TE and ME a single specimen was misclassified (thus resulting, in 90% of correct classifications). Most misclassifications were associated with *C. edule* collected in Albufeira lagoon (AL) and RAl, with respectively 20 and 40% of the specimens collected being erroneously assigned to other ecosystems (Table 11).

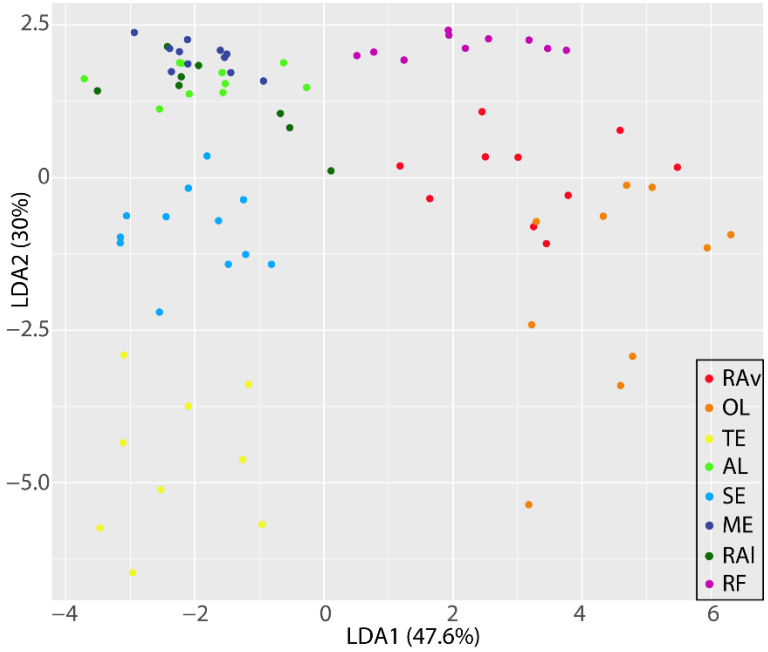

Figure 24. Linear discriminant analysis (LDA) of cockles based on trace elements fingerprints of shells collected from eight different ecosystems along the Portuguese coast: Ria de Aveiro (RAv), Óbidos Lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

Table 11. Classification success (by ecosystem) of a linear discriminant analysis (LDA) for *Cerastoderma edule* shells based on trace element fingerprints. Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF).

	% Predicted Ecosystem								Total per ecosystem	% correct (ecosystem)
	RAv	OL	TE	AL	SE	ME	RAl	RF		
Original Ecosystem										
RAv	100	0	0	0	0	0	0	0	10	100
OL	0	100	0	0	0	0	0	0	10	100
TE	0	0	90	0	10	0	0	0	10	90
AL	0	0	0	80	10	0	10	0	10	80
SE	0	0	0	0	10	0	0	0	10	100
ME	0	0	0	0	0	90	10	0	10	90
RAl	0	0	0	20	0	20	60	0	10	60
RF	0	0	0	0	0	0	0	100	10	100
Average classification success										90

In RAv, TEF of *C. edule* shells showed that, with the exception of Mg/Ca, all ratios (Mn/Ca, Ba/Ca and Sr/Ca) increased from 2013 to 2014 (Figure 25). As a significant interaction between time x areas was recorded for all ratios (MANOVA, F = 8.24, $p < 0.0001$) and significant differences between areas were also recorded for each year (MANOVA, F = 9.44, $p < 0.0001$), the analysis of TEF of *C. edule* shells was made separately for each area to avoid the masking of any potential inter-annual differences. This analysis revealed that Mg/Ca, Mn/Ca, Ba/Ca and Sr/Ca, varied significantly between 2013 and 2014 in all areas, with exception of areas E and M2 for Mn/Ca and Ba/Ca, respectively (Figure 25). TEF of *C. edule* shells was analysed between areas for each year.

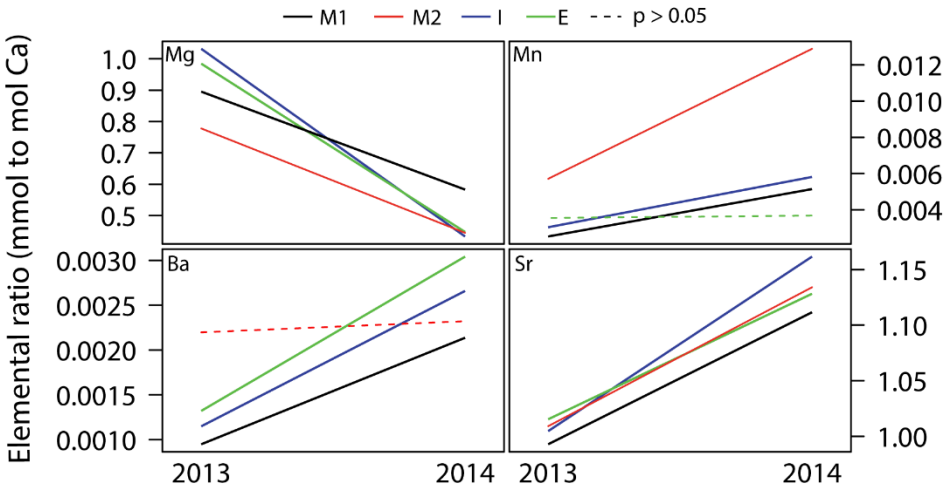

Figure 25. Evolution of elemental ratios of Mg, Mn, Ba and Sr in trace elements fingerprints (TEF) of cockle shells from 2013 to 2014 in areas: Mira Channel (M1 and M2), Ílhavo Channel (I) and Espinheiro Channel (E). The dotted lines represent significant differences in the elemental ratio between years ($p < 0.05$).

In 2013, Ba/Ca, Mn/Ca and Mg/Ca showed significant differences between area M2 and all other areas, except M2 and M1 for Mg/Ca (Figure 26). In 2014, Mg/Ca was the sole element that revealed significant differences between area M1 and all other areas. The ratio of Ba/Ca was significantly different between area I and areas M1 and M2, whereas the Sr/Ca was significantly different between area E and areas M1 and I (Figure 26).

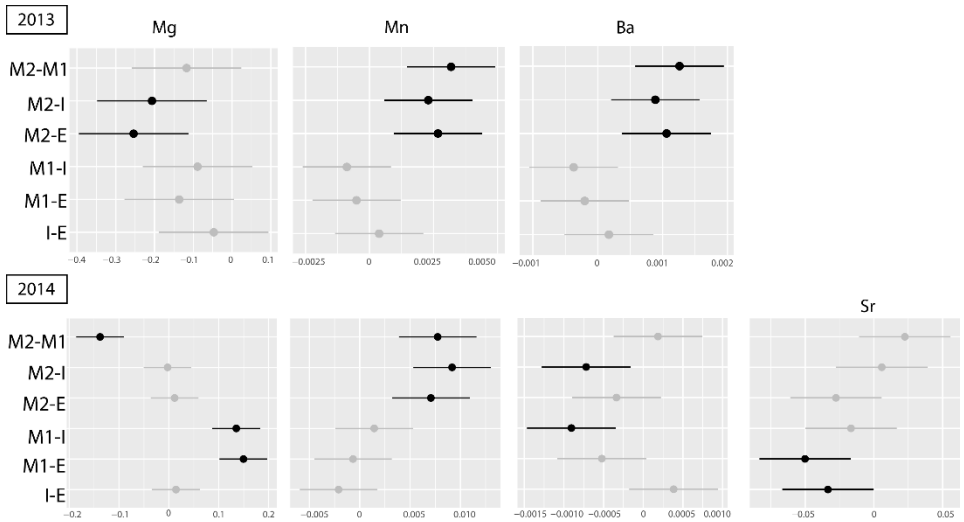

Figure 26. Tukey plot with black lines indicating significant differences in elemental ratios of trace elements fingerprints in cockle shells among areas within Ria de Aveiro (ANOVA, Tukey test comparisons, $p < 0.05$) over two consecutively years (2013 and 2014).

3.2.4. Discussion

Trace element fingerprints (TEF) displayed by cockle shells exhibited significant differences among ecosystems, most likely due to the prevalence of highly dynamic biogeochemical processes such as (e.g. in estuaries and coastal lagoons). The recurrent shifting of environmental conditions (e.g. salinity, temperature, rainfall) in these ecosystems promotes more or less dramatic changes in water chemistry, which are ultimately reflected in the TEF (Chang & Geffen, 2013; Elsdon, et al., 2008) of specimens colonizing these habitats. Indeed, the ratios of the trace elements monitored in the present study (Mg/Ca, Mn/Ca, Sr/Ca and Ba/Ca) have already been reported to display significant spatio-temporal variations in the shells of bivalves as a consequence of shifting environmental conditions (Cathey, Miller, & Kimmel, 2014; Ricardo et al., 2015b). Hence, as also confirmed by the present study, TEF of bivalve shells are not likely to remain stable over two consecutive years (Carson, 2010; Cathey, Miller, & Kimmel, 2014; Zacherl, 2005).

In the present study, cockle shells from each ecosystem displayed contrasting TEF, the sole exception being those originating from Mira estuary (ME) and Ria de Alvor (RAl) (Table 10). Nevertheless, linear discriminant analysis (LDA) results showed that, over three-quarters (90%) of cockles were correctly classified to their areas (Table 12). This result highlights the need to combine different statistical tools (other than analysis of variance) when aiming to use

biogeochemical signatures to assign a given specimen to a certain geographic origin. The results reported in the present study to successfully allocate a sampled specimen to its geographic origin are higher than those reported in previous studies addressing other bivalves (mussel species) along the coast of California (56%) (Becker, Fodrie, McMillan, & Levin, 2004) and the Gulf of Maine (68%) (Sorte, Etter, Spackman, & Hannigan, 2013). Such an accurate identification of geographic origin as the one achieved for *C. edule* in along the Portuguese coast is likely to be due to a combination of several factors, such as anthropogenic pressure (influencing Mn/Ca) (Vale, Botelho, Rodrigues, Gomes, & Sampayo, 2008) and an increased input level of trace elements originating from terrestrial runoff (e.g. Mn/Ca and Ba/Ca) (Thébault et al., 2009) and their preferential retention in estuaries and coastal lagoons. It is also worth highlighting that the incorporation of trace elements may be significantly affected by shifting water temperature (affecting Mg/Ca and Sr/Ca) (Klein, Lohmann, & Thayer, 1996) and salinity (affecting all ratios determined in this study) (Poulain et al., 2015), which due to the distinctive morphodynamics of each of the ecosystems surveyed may shift in a number of unique patterns and combinations. The higher ratios of Mg/Ca recorded in cockle shells from specimens in the northernmost locations surveyed (Ria de Aveiro (RAv) and Óbidos lagoon (OL)) are in accordance with the latitudinal temperature gradient displayed by this element in seawater (Poulain et al., 2015; Schöne et al., 2011). It is also worth highlighting that such differences may also be associated with more or less pronounced shifts in water temperature promoted by the size and shape of the estuarine systems surveyed (e.g. small and large estuaries and coastal lagoons).

The highest concentrations of Ba/Ca in TEF is often derived from freshwater inputs and nutrient runoffs to estuarine systems, which are known to promote an increase in primary productivity (Lazareth, Putten, André, & Dehairs, 2003; Thébault et al., 2009; Putten, Dehairs, Keppens, & Baeyens, 2000). It is therefore possible that the runoffs originating from the fertile lands in the margins of RAv, OL and Ria Formosa (RF) that are used for agricultural purposes may contribute to the occurrence of diatom blooms that can increase the availability of Ba (Thébault et al., 2009). Concerning the significantly higher levels of Mn/Ca present in cockle shells originating from Tagus estuary (TE) it is likely associated with the legacy of former anthropogenic actions on the sampling site (namely historical metal industries), which has promoted the build-up of trace metals on surface water and sediment (Vale, Botelho, Rodrigues, Gomes, & Sampayo, 2008).

Estuaries are dynamic environments in which coastal fluxes of trace elements and mixing rates with seawater vary from a tidal to an annual time scale (Peters, 1999). In this way, it was not surprising to record significant shifts in the TEF of cockle shells of adult specimens originating from 4 different areas in RAv over two consecutive years. Indeed, Cathey, Miller, & Kimmel (2014) had already reported significant temporal variability (tri-weekly) in the TEF of larval bivalve shells being cultured in different hatcheries employing estuarine water in their

operation in the southern Delmarva Peninsula (Virginia, USA). Carson (2010) also refers the occurrence of inter-seasonal and inter-annual variation in the TEF of Olympia oyster (*Ostrea lurida*) shells sampled in four different estuaries in Southern California (USA). Nonetheless, it is important to highlight that although larval and young juvenile TEF of bivalve shells may significantly shift in a weekly basis (Becker, Fodrie, McMillan, & Levin, 2004; Fodrie, Becker, Levin, Gruenthal, & McMillan, 2011), older juveniles are known to display stable TEF over a six-month period (Dunphy, Millet, & Jeffs, 2011). Within the scope of using TEF of bivalve shells to verify claims on the geographic origin of adult live specimens traded for human consumption, temporal variability likely not be an issue if samples to verify the claim can be collected from the same area of the specimens being traded. These TEF will certainly match, as the time scale of shelf-life for live bivalves is measured in days and not weeks or months. However, if this matching is performed using the TEF of bivalves originating from the same area but analyzed more than a year ago (e.g. by using data from a TEF database), temporal variability may significantly bias the analysis and an erroneous assignment of geographic origin is likely to occur. This pitfall associated with temporal variability is less likely to take place when comparing specimens originating from different ecosystems (Carson, 2010).

Overall, the present study reinforces the potential that the TEF of cockle shells hold to be used as proxies for inferring the place of origin in traceability frameworks. Nonetheless, the analytical costs associated with the determination of TEF in bivalve shells whenever the need arises to verify claims related with their geographic origin can rapidly become prohibitive. In this scenario, it may be possible to rely on TEF previously recorded for specimens originating from the area being claimed by the producer/collector/trader. The time window during which this comparison can be performed without being prone to bias caused by temporal variability is likely to span between six months and less than a year. Future studies should try to determine as accurately as possible the span of such time window, as well as verifying if it does not change among capture/production areas.

CHAPTER 4. CONCLUDING REMARKS

The biochemical and geochemical tools tested during the present study can open a window of opportunity to enhance the economic benefits associated with the trade of bivalves. The transfer of this technology to bivalve producers and traders can allow them to add value to their products by allowing their differentiation and enhancing their promotion near end consumers. Moreover, these tools may also be employed against fraud, namely when enforcing food safety issues. Overall, the methodologies employed reveal the potential they hold for aggregating an even higher market value for products that continue to be perceived by society as a synonym of health and quality. There is a window of opportunity for seafood in general, and bivalves in particular, to achieve an even higher commercial revenue if they are traded in niche markets that value origin certification of traded products. The use of biochemical and geochemical tools will enable fishermen/producers to achieve a certification of origin of their products, allowing different stakeholders in the value chain, namely the final consumer, to trace these products "from farm to fork".

The transfer of the present technology to national (and international) authorities will allow a better monitoring of trade chains, as it will allow the exposure of fraudulent practices involving inaccurate claims on the geographic origin of traded specimens. The use of biochemical and geochemical signatures can be used to differentiate seafood products under different scenarios such as:

- allowing bivalve nurseries to track the seeds supplied to producers to manage conflicts resulting from claims on bivalves mortality;

- denouncing the trade of bivalves labelled as originating from a specific capture location where harvesting is allowed when in fact those specimens originate from a region where harvesting is interdicted for any food safety reason (e.g. occurrence of harmful algal blooms, high microbial loads, metal contamination).

Product valorisation can be achieved through differentiation and by promoting an active fight against fraud. This will pave the way for expansion to new markets of added value, where producers and traders can obtain a higher for premium and safe products. The present study contributes to the development of innovative traceability tools that add value to seafood, contribute to Blue Growth and facilitate strategies that promote the Economy of the Sea in national territories and abroad.

CHAPTER 5. REFERENCES

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Appendix A. Study areas

Ria de Aveiro

Ria de Aveiro is located on the northwestern coast of Portugal, between 40° 38`N and 40º 57`N. This system is a shallow and well mixed coastal lagoon, characterized by extensive intertidal mud and sand flats, salt marshes and islands, forming four main Channels, Mira, Ílhavo, Espinheiro and São Jacinto-Ovar. The only connection between Ria's and the Atlantic Ocean is made through an artificial inlet with 1.3 km length, 350 m wide and 20 m depth, constructed in 1808. Hydrodynamically, Ria the Aveiro is a mesotidal lagoon, characterized by semidiurnal tides, which are the main forcing agent driving water circulation in the lagoon (e.g. Dias, Lopes, & Dekeyser, 2000; Lopes, Vaz, Vaz, Ferreira, & Dias, 2015). In the upper reaches of the channels, the tidal time delay relatively to the mouth is about 6 h. The total catchment area of the Ria de Aveiro is 3500 km^2 of which 80% is drained by the River Vouga (Stefanova, Krysanova, Hesse, & Lillebø, 2015). Rivers Vouga at the Espinheiro channel and Antuã at the Laranjo basin are responsible for the major fluvial inputs. The Boco river, at the southern end of Ílhavo channel, and the Cáster and the Gonde rivers, discharging at the north end of São Jacinto channel, present a negligible flow. At the southern end of Mira channel the input of freshwater corresponds to a small system of ponds and rivers. Salinity in Ria de Aveiro is influenced by the combined effects of freshwater discharges and tidal penetration, exhibiting a longitudinal gradient of salinity from about 0 in freshwaters at upstream areas, to about 36 at the Ocean boundary.

Figure S1. Ria de Aveiro, northwestern coast of Portugal (40° 40′ 37″ N, 8° 40′ 28.90″ W).

Óbidos lagoon

The Óbidos lagoon is located on the north-western coast of Portugal, covering an area between 4.4 km^2 at mid-tide and 8 km^2 at high-tide, with an average depth of 3 m. It is separated from the sea by a 1 km long sand spit, interrupted by a 100 m wide wandering inlet. Tidal amplitude ranges from 2 to 4 m at the coastal zone and 1 to 2 m inside the lagoon (Oliveira, Fortunato, & Rego, 2006). Freshwater enters the lagoon through the Barrosa and Bom Sucesso arms, being negligible mainly in Spring/Summer (Rodrigues, Quintino, Pereira, & Freitas, 2012).

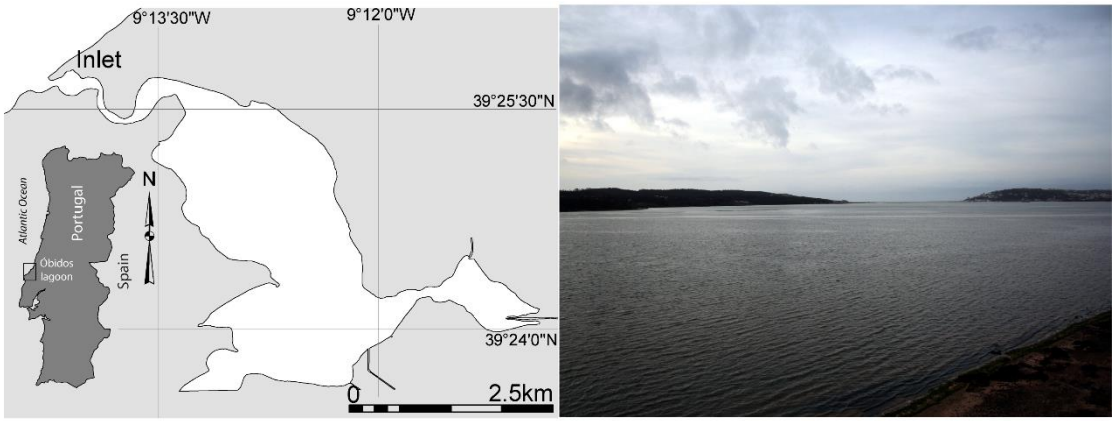


Figure S2. Óbidos lagoon, western coast of Portugal (39º23'18" N and 9º13'34" W).

Tagus estuary

Tagus estuary, western coast of Portugal, has a broad shallow bay covering an area of 320 km^2 (Brogueira & Cabeçadas, 2006) from which, 110 km^2 corresponds to intertidal areas, 20 km^2 are occupied by salt marsh vegetation and 80 km^2 by mudflats (http://www.maretec.mohid.com/portugueseestuaries/tagus/Tagus.htm). The main freshwater input comes from the Tagus River, a water body with an annual average riverine flow of 400 $m^3 s^{-1}$ (Santos-Echeandía, Caetano, Laglera, & Vale, 2013). Seawater enters the estuary through a deep narrow inlet channel and, according to the NOAA classification, Tagus is a mesotidal estuary, with semi-diurnal tides ranging from 0.4 m to 4.1 m at neap and spring tides, respectively (Duarte, Caçador, Marques, & Croudace, 2013). The average depth is < 10 m and salinity varies from 0 upstream to nearly 37 at the estuary mouth (França, Vinagre, Caçador, & Cabral, 2005). Tagus estuary is surrounded by the Lisbon metropolitan area, receiving effluents from agricultural, industrial and urban sources (Gameiro & Brotas, 2010). Tagus estuary presents high levels of contamination due to direct discharges of urban and industrial effluents into the system (Vasconcelos et al., 2007). Industry (e.g. chemical, petrochemical, metallurgic, shipbuilding and cement plants) represents a significant anthropogenic pressure on this area, especially taking into account the existing 18000 industries with a corresponding industrial load of 75.5×10^6 m^3 (Vasconcelos et al., 2007). As a consequence, besides other kid of contaminants,

contamination by metals are particularly relevant (Caçador et al., 2012), reaching values 20 times higher comparatively with the natural background (Figueres, Martin, Meybeck, & Seyler, 1985).

Albufeira lagoon

The Albufeira lagoon is located on the western Portuguese coast, about 20 km South of Lisbon. The lagoon extends perpendicularly to the coast over 3.6 km, covering an area of 1.3 km^2, with a maximum depth of 13 m. The lagoon is made up of two basins separated by a narrow and shallow channel. Lagoa Grande is the main body, with an average depth of about 10m, although it may reach 20 m due to abundant winter rain. In the smaller body, called Lagoa Pequena, the average depth is about 2 m (Alday, Cearreta, Freitas, & Andrade, 2013). The lagoon is sheltered from the Atlantic Ocean by a narrow littoral barrier with 1.2 Km long and a mixture of mobile shallow channels and sandbanks. In front of the lagoon, tides are semi-diurnal and the tidal range varies between 0.55 and 3.86 m (Alday, Cearreta, Freitas, & Andrade, 2013). The Albufeira lagoon connects with the Atlantic Ocean through an inlet inserted into a 24 km long beach, between the mouth of the Tagus estuary and the Espichel Cape. The process of artificial barrier breaching is well documented in historical records since the 15^{th} century (Freitas & Andrade, 1994) and, at present, is done on an annual basis in order to ensure the water quality. The occupation of the alluvial plains in the main tributaries for agriculture, the presence of mussel aquaculture and the discharge of urban effluents into the lagoon created a permanent need to control the water level and quality (Alday, Cearreta, Freitas, & Andrade, 2013). The physical-chemical characteristics of the water are mainly controlled by the exchanges between the lagoon and the sea, and by the amount of freshwater input (Freitas & Andrade, 1994).

Sado estuary

The Sado estuary is located on the western coast of Portugual, 40 km south of the intensive industrialised and populated metropolitan area of Lisbon (Costa, Marques, Freitas, Reis, & Oliveira, 2002). This ecosystem covers an area of about 180 km^2, corresponding to the second largest estuary in Portugal. It has large intertidal areas (78 km^2) (Vasconcelos et al., 2007) and two channels in the lower estuary, separated by a set of sand banks and 3 main basin in the upper part (Marateca, Alcácer Channel and Comporta). Sado River contributes with 80 to 90% of the freshwater inflow through Alcácer Channel with average annual pluvial discharge of 10 $m^3 s^{-1}$. Marateca Stream contributes with approximately 10% of the freshwater input with a flow of about 1 $m^3 s^{-1}$. Tides are semi-diurnal, with a tidal range of 1.6 m in spring tides and 0.6 m in neap tides (Martins, Leitão, Silva, & Neves, 2001).

The system presents a relevant ecological importance and also a considerable socio-economic value due to industry, aquaculture, salt production, fishing and recreational activities (Freitas et al., 2011; Rodrigues, Bio, Amat, & Vieira, 2011). Sado estuary supports the

industrial zone of the Setubal city located at its northern part, responsible for high anthropogenic pressures (Caeiro, Goovaerts, Painho, & Costa, 2003; Martins, Ferreira, & Vale, 2008). Besides, history of mining activities and pyrite outcrop erosion have contributed to the input of metals, namely Cd, Zn, Cu and locally Hg and Pb (Quevauviller, Lavigne, & Cortez, 1989).

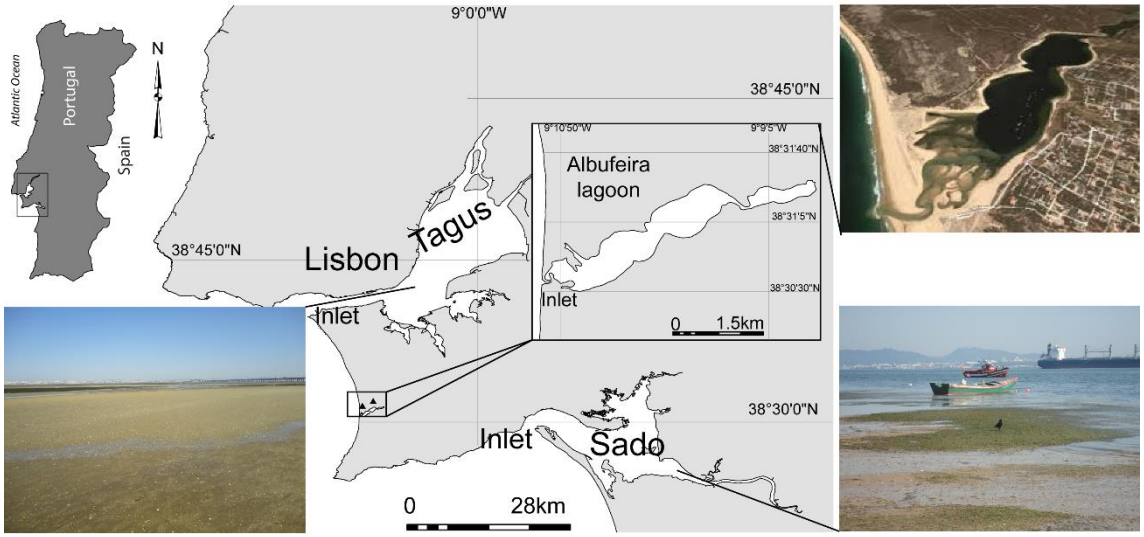


Figure S3. Tagus estuary (38º39'27" N and 9º6'36" W), Albufeira lagoon (38º30'24" N and 9º10'52" W) and Sado estuary (38º31'14" N and 9º53'32" W), western coast of Portugal.

Mira estuary

The Mira estuary is a narrow entrenched estuary located at the southwestern coast of Portugal, between Vila Nova de Milfontes at the mouth and Odemira at its upper limit. The estuary is approximately 40 km long and 400 m wide near its mouth (Blanton, Ferreira, & Andrade, 2000). The mean depths are of about 1.2 m and 8 m upstream and near the river mouth, respectively, and the residence time is of fourteen days. This ecosystem comprises an area of 2.85 km^2 of salt marsh, of which 2.5 km^2 have been proposed for reclamation for aquaculture. Fifty years ago, an agriculture reservoir (Santa Clara) was constructed 50 km upstream of the river mouth, influencing the freshwater flow regime (Blanton, Ferreira, & Andrade, 2000).

Mira estuary is included in a nature protected area (Parque Natural do Sudoeste Alentejano e Costa Vicentina) and the level of anthropogenic pressure is reduced and much lower than in other Portuguese estuaries (Bettencourt et al., 2004). There are no large urban and industrial areas near this system so, it is less exposed to nutrient and chemical pollution. However, there are some intensive agriculture units, cattle breeding, aquaculture activities and domestic sewage discharge (Castro & Freitas, 2006). The upstream estuarine areas show a slight contamination by heavy metals due to previous mineral extraction activities in the river basin

Figure S4. Mira estuary, southwestern coast of Portugal (37º43'31" N and 8º46'15" W).

Ria de Alvor

The Ria de Alvor is a lagoon system located on Lagos Bay, on the south coast of Portugal, covering an area of approximately 15 km^2. The Ria is separated from Lagos Bay by two barrier peninsulas with sand dunes and connects to the Atlantic Ocean by a single inlet which has been stabilized by two breakwaters constructed in 1990. The system has two main freshwater inputs, the Arão and Odiáxere Rivers, strongly affected by seasonal variation, with torrential flows in wet months and dry in summer months (Quintino & Rodrigues, 1989). Outside of the navigation channel, which is dredged to maintain navigability to the recreational and fishing port of Alvor, the maximum depth of the Ria is about 2 m, depending on the tide. During high tide, around 3 km^2 of the Ria (including intertidal areas) are flooded and, at low tide, the surface area of the residual water is approximately of 1 km^2, mostly confined to the inner channels and creeks. Given these conditions, the water is almost entirely renewed at each tide in the outer part of the Ria, resulting in strong tidal currents (Quintino & Rodrigues, 1989). The relatively low freshwater input, associated with the tide dominated hydrodynamic regime, result in a salinity range from 30 to 40.

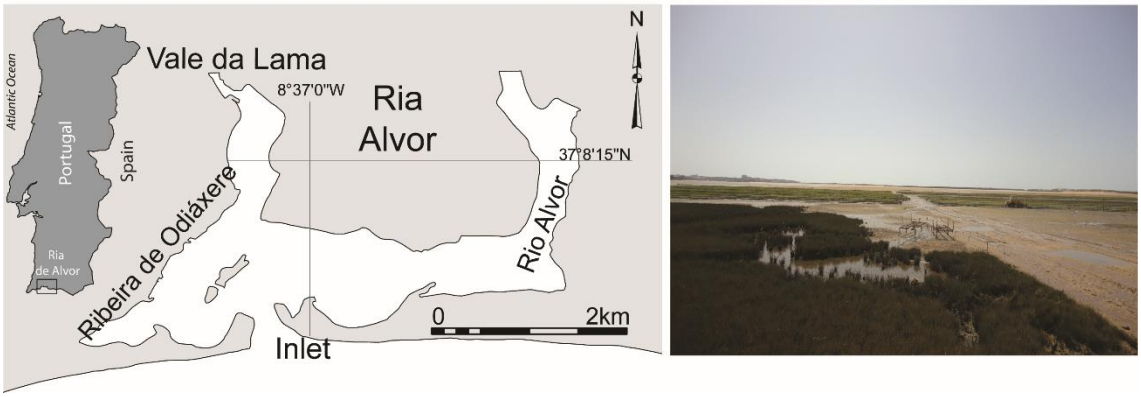


Figure S5. Ria de Alvor, south coast of Portugal (37º7'56" N and 8º37'27" W).

Ria Formosa

The Ria Formosa, located at the south coast of Portugal, is a shallow mesotidal lagoon with 55 km long and 6 km wide, and a mean depth of 3 m. It is a system of barrier islands which communicates with the Atlantic Ocean by five natural and mobile inlets and two deeper ones, maintained through dredging and channel reinforcement (Mudge, Bebianno, East, & Barreira, 1999). Tides are semi-diurnal with amplitudes ranging from 0.7 m to 3.5 m on neap tides and spring tides, respectively, resulting in an exchange of the lagoon water within a few tidal cycles of 50%-75% (Bebianno, 1995). The inner regions of the lagoon are less effected by the tides. Salinity of the Ria Formosa ranges from 32 to 38 and temperature varies between 16 ^{0}C in winter and 21 ^{0}C in summer (Newton & Mudge, 2003). Five small rivers and fourteen streams flow to the lagoon, most of them completely dry out in summer (Dias, Sousa, Bertin, Fortunato, & Oliveira, 2009; Dionisio, Rheinheimer, & Borrego, 2000; Newton & Mudge, 2003).

Ria Formosa consists of 145 km^2 of wetland and 4 km^2 of salt extraction and mariculture ponds, salt marsh, exposed sands and mud banks. This lagoon is a very productive system, with high nutrient concentration levels, insolation and good tidal water exchange (Newton & Mudge, 2005). For many aquatic species it is an important spawning and nursery area, including bivalves, due to its sheltered conditions. These circumstances gave rise to the fishing and mariculture industries, economically very important to the region.

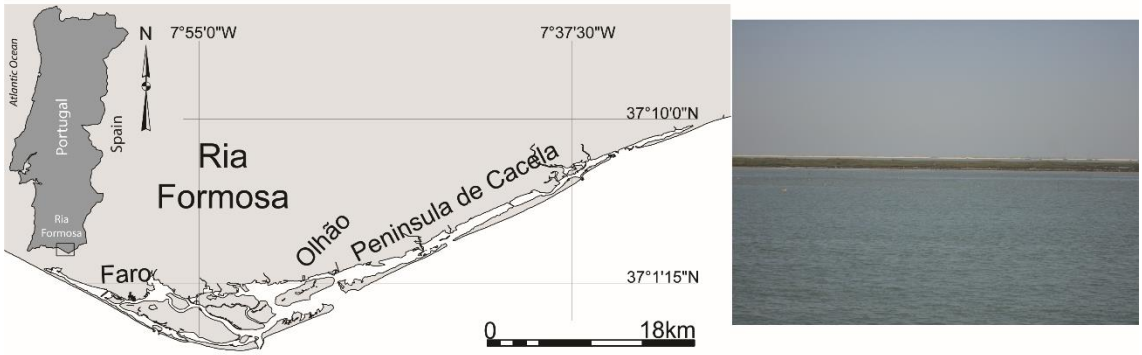


Figure S6. Ria Formosa, south coast of Portugal (37º0'23" N and 7º59'28" W).

Appendix B. Supplementary data of section 2.1

SUPPLEMENTARY TABLES

Table S1. Water salinity (S) and linear distances (in km) between the inlet of Ria de Aveiro and sampling locations in São Jacinto (SJ), Mira (M), Ilhavo (I) and Espinheiro (E) channels. Please note that a lower distance to the inlet may not always mean that the location displays a higher water renewal rate or "more marine/less brackish" conditions, as these features are regulated by the geomorphology of the coastal lagoon and consequently its dominant currents.

Inlet (S 36)								
2.2	**SJ1** (S 36)							
7.6	4.8	**SJ2** (S 35)						
2.8	2.1	6.9	**M1** (S 36)					
9.1	8.6	13.4	6.4	**M2** (S 35)				
7.9	7.2	11.9	4.8	11.5	**I1** (S 32)			
11.6	10.2	15.0	8.3	15.0	3.2	**I2** (S 30)		
6.3	5.4	10.2	3.3	10.0	3.5	6.6	**E1** (S 32)	
8.4	7.8	12.5	5.5	12.1	5.7	8.9	2.4	**E2** (S 30)

Table S2. Similarity values (ANOSIM) between the FA class profiles of the *Cerastoderma edule* adductor muscle from areas within São Jacinto, Mira, Ilhavo and Espinheiro Channels, Ria de Aveiro, Portugal.

Channels	**R**	***p***
São Jacinto 1 vs São Jacinto 2	0.271	0.114
Mira 1 vs Mira 2	0.013	0.405
Ilhavo 1 vs Ilhavo 2	0.081	0.222
Espinheiro 1 vs Espinheiro 2	0.116	0.190

Appendix C. Supplementary data of section 2.2

SUPPLEMENTARY TABLES

Table S3. Multivariate analysis of variance (MANOVA) among groups of fatty acids of the adductor muscle of live common cockles *Cerastoderma edule* from areas within Mira (M1 vs M2), Ilhavo (I1 vs I2) and Espinheiro (E1 vs E2) Channels from Ria de Aveiro (RAv), Óbidos lagoon (OL1 vs OL2), Tagus estuary (TE1 vs TE2), Albufeira lagoon (AL1 vs AL2), Sado estuary (SE1 vs SE2) and Ria Formosa (RF1 vs RF2), Portugal.

Area	df	pillai	approx. F	p. value
M1 vs M2	1	0.991	14.1	0.203
I1 vs I2	1	0.684	0.62	0.735
E1 vs E2	1	0.894	2.42	0.323
OL1 vs LO2	1	0.926	6.27	0.080
TE1 vs TE2	1	0.926	6.30	0.079
AL1 vs AL2	1	0.991	14.10	0.203
SE1 vs SE2	1	0.944	8.44	0.540
RF1 vs RF2	1	0.659	0.97	0.558

Table S4. Fatty acid profiles (data presented as % of relative abundances) of the adductor muscle of live common cockles *Cerastoderma edule* (values are means of 10 replicates ± SD) from Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF), Portugal. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; and HUFA – highlyunsaturated fatty acids.

Fatty Acid	**Ecosystem**							
(%)	**RAv**	**OL**	**TE**	**AL**	**SE**	**ME**	**RAl**	**RF**
14:0	1.33±0.44	1.21±0.33	1.22±0.24	1.56±0.63	1.59±0.57	1.57±0.16	1.72±0.36	1.81±0.30
15:0	0.41±0.10	0.54±0.19	0.54±0.14	0.75±0.25	0.39±0.07	0.52±0.06	0.50±0.07	0.49±0.06
16:0	12.03±1.61	13.69±1.33	14.22±1.73	11.02±2.75	11.02±1.97	12.28±1.10	11.08±0.81	12.62±1.48
17:0	1.61±0.19	1.05±0.28	1.69±0.27	2.06±0.53	2.57±0.94	2.00±0.22	1.74±0.29	1.63±0.25
18:0	12.33±1.23	12.21±0.73	12.92±2.26	10.69±2.54	12.49±1.60	12.86±1.36	11.86±1.05	10.44±1.05
SFA	**27.70±3.56**	**28.71±2.88**	**30.59±4.64**	**26.08±6.70**	**28.07±5.15**	**29.22±2.91**	**26.89±2.56**	**26.99±3.16**
16:1*n*9	2.02±0.23	1.92±0.36	2.26±0.40	2.23±0.60	1.90±0.58	2.65±0.82	1.99±0.27	2.40±0.55
18:1*n*9	1.44±0.27	5.82±3.74	2.75±1.81	6.45±6.25	3.18±3.19	2.32±1.24	1.72±0.18	8.01±5.72
18:1*n*7	2.88±0.42	3.12±0.56	3.18±0.56	2.26±0.42	3.46±0.56	2.96±0.44	3.01±0.49	3.12±0.40
20:1*n*9/*n*11	3.41±0.31	3.89±1.24	3.93±0.92	4.84±1.01	3.27±0.34	3.40±0.67	3.43±0.19	3.69±0.73
20:1*n*7	2.72±0.58	3.20±0.49	2.87±0.40	2.71±0.32	2.99±0.39	2.53±0.15	2.97±0.42	2.04±0.32
MUFA	**12.47±1.82**	**17.94±6.39**	**15.00±4.08**	**18.49±8.61**	**14.80±5.05**	**13.86±3.31**	**13.11±1.55**	**19.26±7.72**
18:2*n*6	0.84±0.13	3.26±2.56	1.31±0.67	2.73±2.22	1.77±2.37	1.43±0.60	1.14±0.16	3.45±2.09
20:2*n*9	1.06±0.20	1.41±0.26	1.32±0.21	1.02±0.21	0.88±0.19	1.18±0.19	1.17±0.16	1.34±0.20
22:2*n*9	3.91±0.76	2.90±0.54	3.60±1.02	4.06±1.16	3.52±0.68	3.21±0.56	3.77±0.68	3.28±0.77
22:2*n*6	2.06±0.43	2.10±0.42	2.67±1.21	2.97±0.88	3.29±1.02	2.28±0.59	3.02±1.17	1.83±0.47
22:3*n*6	3.73±0.58	3.73±0.55	3.99±0.93	4.14±0.83	3.48±0.39	3.72±0.37	4.26±0.60	3.77±0.74
PUFA	**11.59±2.09**	**13.39±4.34**	**12.89±4.05**	**14.92±5.31**	**12.94±4.65**	**11.83±2.30**	**13.36±2.77**	**13.66±4.26**
20:4*n*6	3.52±0.58	2.94±0.50	4.23±1.29	6.74±1.13	5.23±0.97	5.67±0.41	4.30±0.45	3.98±0.76
20:5*n*3	14.91±0.69	13.33±2.51	13.36±1.30	10.05±1.24	15.34±1.83	13.84±0.76	14.28±1.03	13.34±2.05
22:4*n*6	1.45±0.53	1.01±0.30	1.96±0.50	2.49±0.68	2.73±0.49	2.54±0.40	2.21±0.46	1.74±0.35
22:4*n*3	1.08±0.15	0.80±0.13	1.71±0.26	1.61±0.38	1.31±0.23	1.33±0.09	1.05±0.07	1.07±0.15
22:5*n*3	3.64±0.92	3.00±0.50	4.20±0.52	3.28±0.52	4.26±0.81	3.18±0.27	4.40±0.51	3.46±0.53
22:6*n*3	22.68±2.52	17.28±3.31	14.84±2.56	14.19±2.80	14.65±2.51	17.07±2.36	19.39±1.23	13.49±1.68
HUFA	**47.27±5.39**	**38.36±7.06**	**40.28±6.43**	**38.35±6.75**	**43.53±6.83**	**43.64±4.29**	**45.63±3.75**	**37.09±5.53**
*n*6/ *n*3	27.52±4.65	39.84±14.53	42.20±12.11	67.00±15.18	47.34±12.47	44.55±6.08	38.41±7.89	47.78±8.86

SUPPLEMENTARY FIGURES

Figure S1a. Significant differences (ANOVA; Tukey plot) among fatty acids 22:4*n*-3, 22:4*n*-6, 22:5*n*-3, 22:6*n*-3, PUFA – polysaturated fatty acids and *n*-3/ *n*-6 ratio present in the adductor muscle of live common cockles *Cerastoderma edule* from Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF). Significant differences ($p < 0.05$) among shelf-life times are highlighted with black lines.

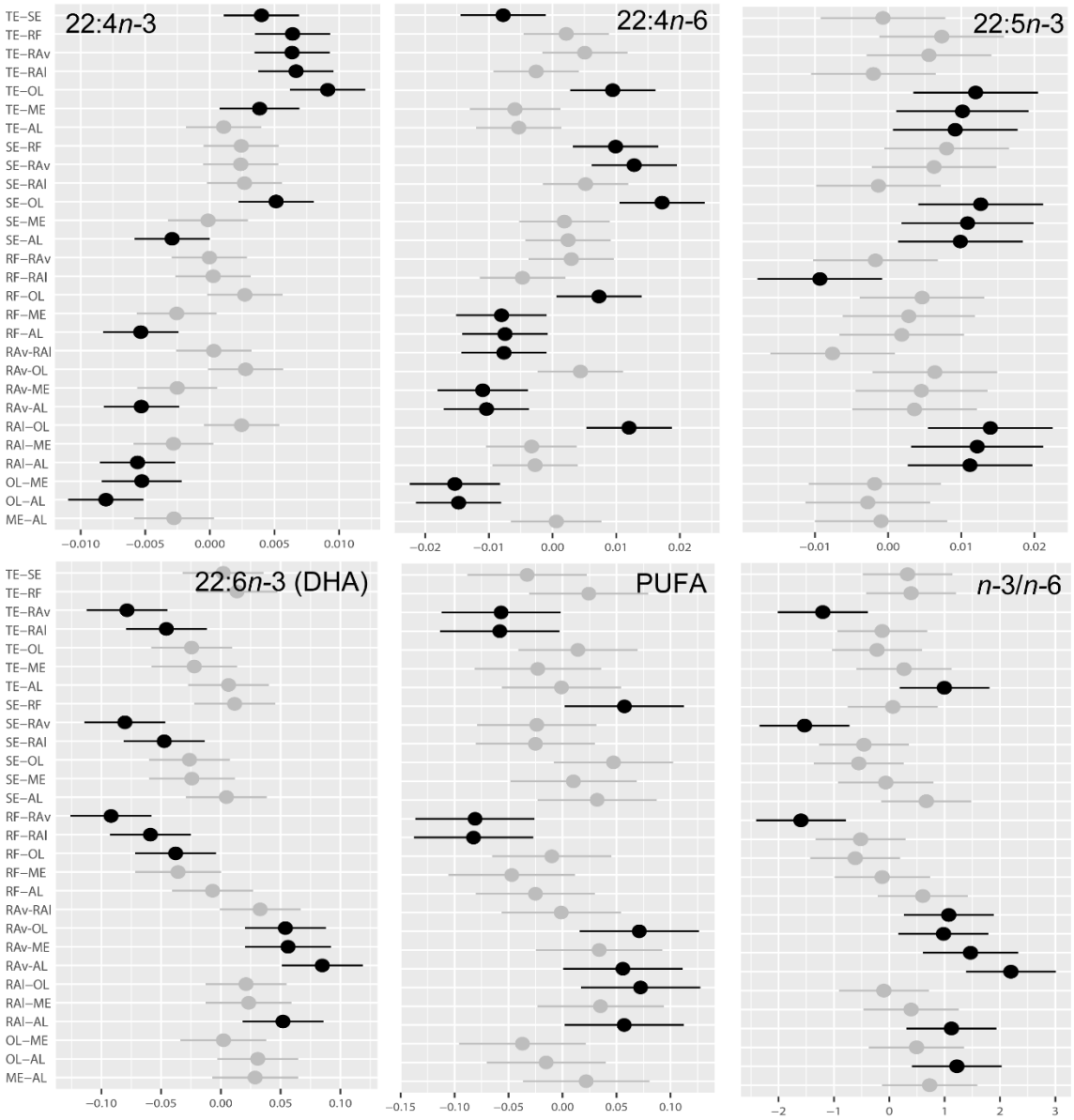


Figure S1b. Significant differences (ANOVA; Tukey plot) among fatty acids 18:2*n*-6, 20:2*n*-9, 22:2*n*-9, 22:2*n*-6, 20:4*n*-6 and 20:5*n*-3 present in the adductor muscle of live common cockles *Cerastoderma edule* from Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF). Significant differences ($p < 0.05$) among shelf-life times are highlighted with black lines.

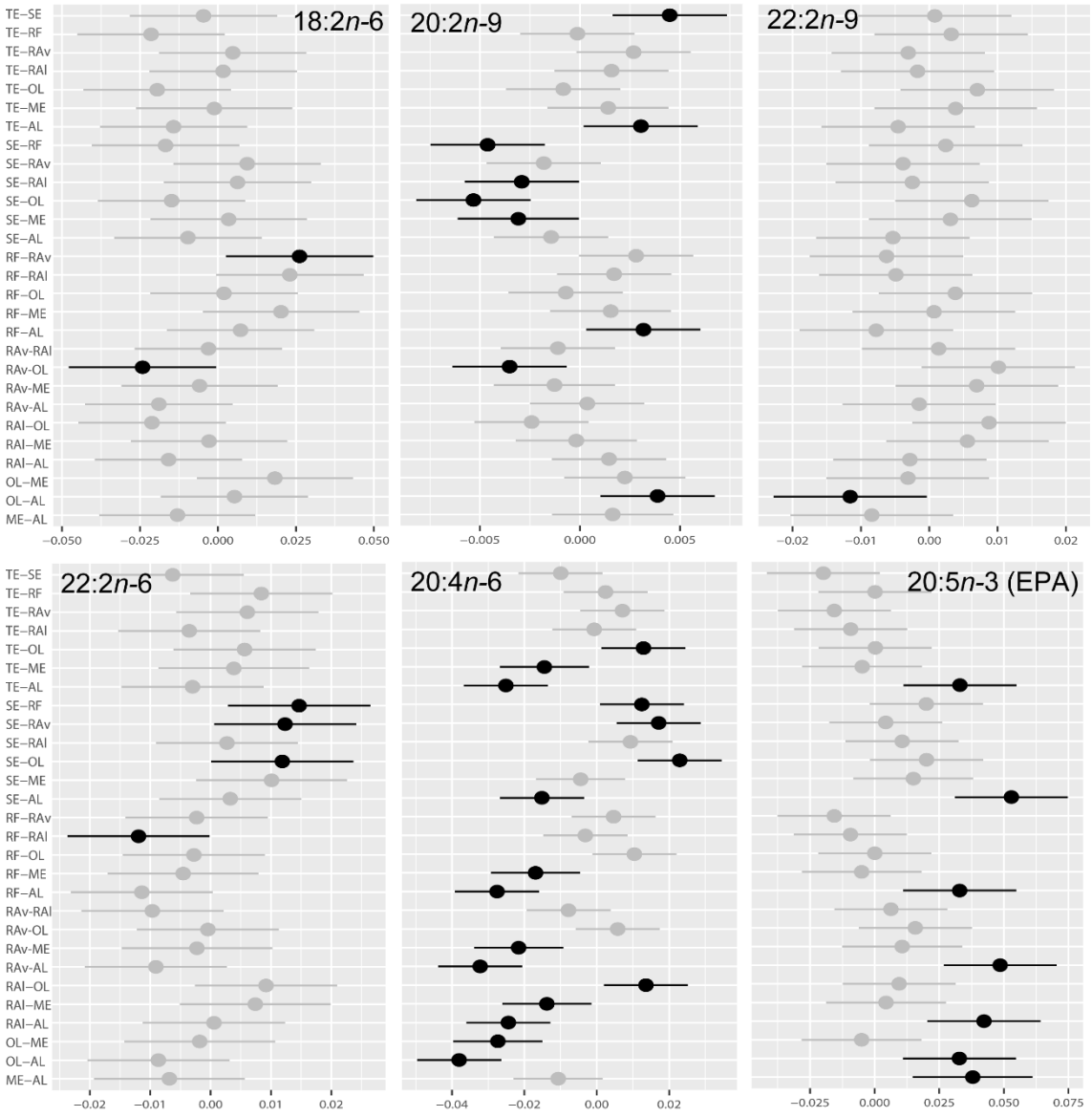


Figure S1c. Significant differences (ANOVA; Tukey plot) among fatty acids 14:0, 15:0, 16:0, 17:0, 18:0 and sum SFA – saturated fatty acids present in the adductor muscle of live common cockles *Cerastoderma edule* from Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF). Significant differences ($p < 0.05$) among shelf-life times are highlighted with black lines.

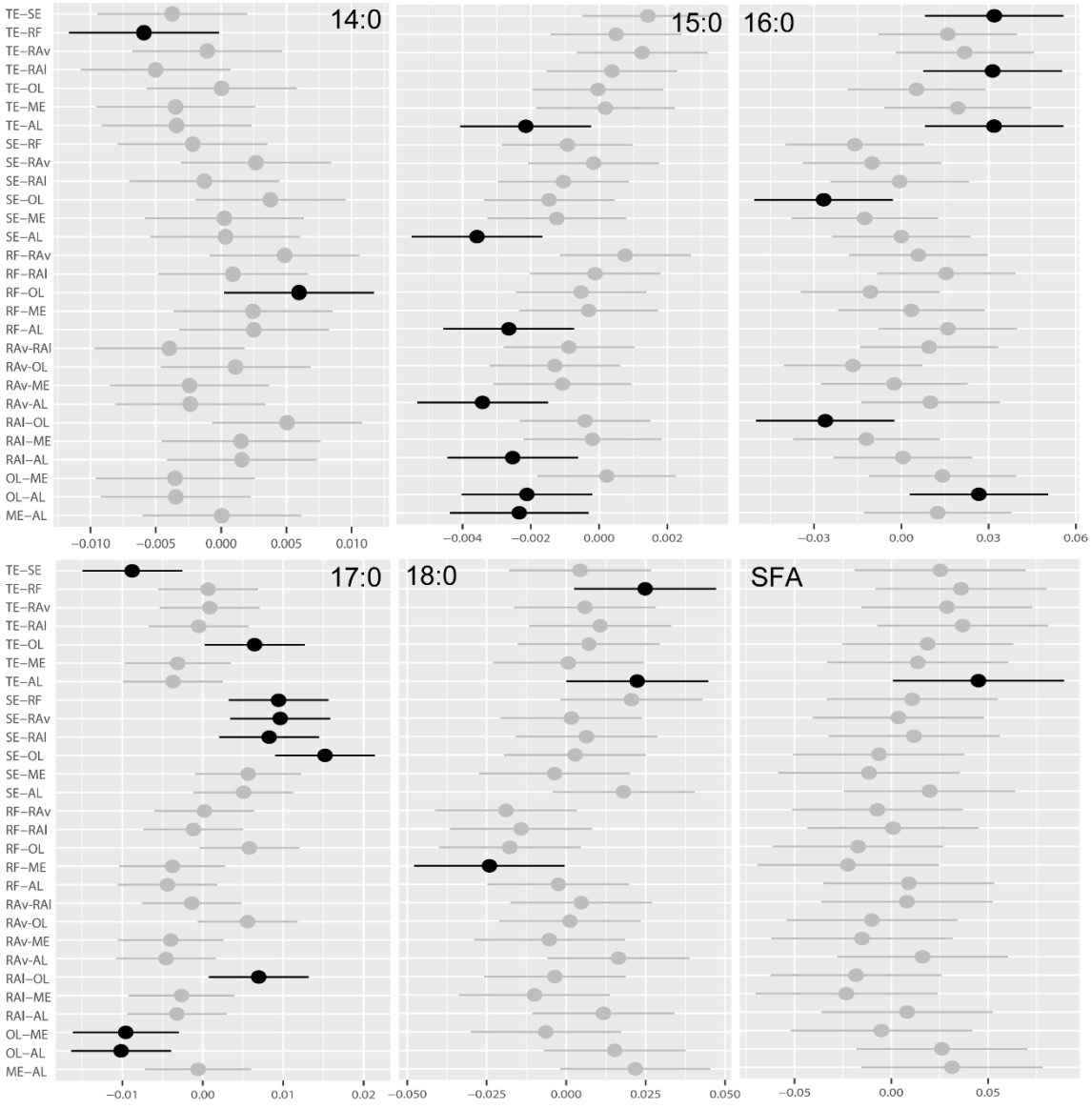
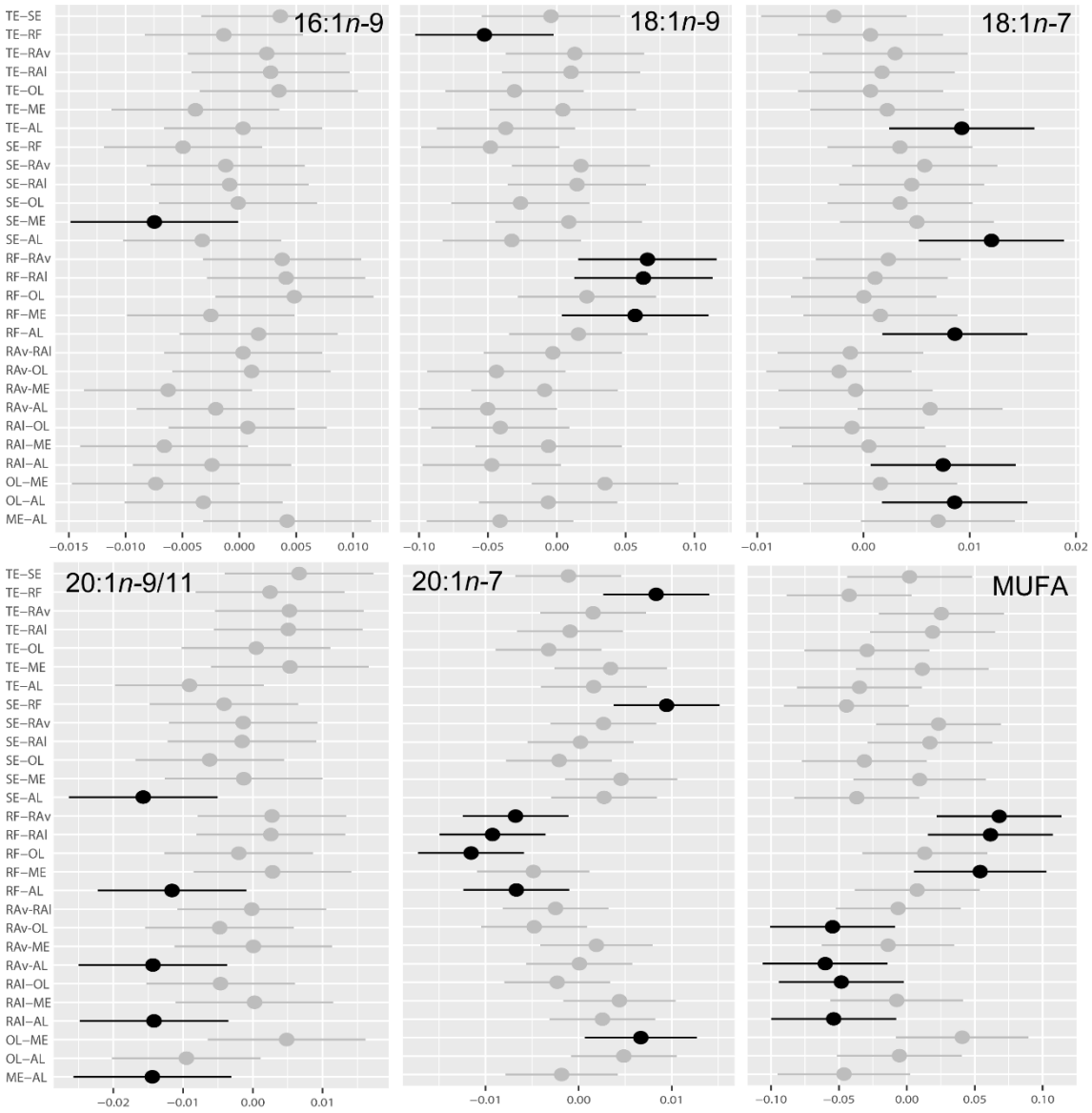


Figure S1d. Significant differences (ANOVA; Tukey plot) among fatty acids 16:1*n*-9, 18:1*n*-9, 18:1*n*-7, 20:1*n*-9/11, 20:1*n*-7 and sum MUFA – unsaturated fatty acids present in the adductor muscle of live common cockles *Cerastoderma edule* from Ria de Aveiro (RAv), Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL), Sado estuary (SE), Mira estuary (ME), Ria de Alvor (RAl) and Ria Formosa (RF). Significant differences ($p < 0.05$) among shelf-life times are highlighted with black lines.



Appendix D. Supplementary data of section 2.3

SUPPLEMENTARY TABLES

Table S5. Fatty acid profiles (data presented as % of relative abundances) of the adductor muscle of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC. Values are means of 5 replicates ± standard deviation. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA –polyunsaturated fatty acids and HUFA – highlyunsaturated fatty acids.

Fatty Acid	**Shelf-life time (days)**							
(%)	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
14:0	2.19±0.26	1.87±0.19	1.76±0.42	1.70±0.50	1.55±0.25	1.71±0.39	1.62±0.36	1.68±0.13
15:0	0.70±0.10	0.66±0.12	0.57±0.13	0.49±0.05	0.51±0.14	0.45±0.04	0.44±0.04	0.46±0.14
16:0	13.59±2.22	13.13±1.76	11.06±0.62	12.65±0.30	9.26±2.81	9.67±1.41	8.97±1.04	8.47±0.72
17:0	1.29±0.18	1.58±0.18	1.65±0.14	1.60±0.09	8.39±7.98	10.84±4.02	8.69±1.54	13.27±2.82
18:0	14.16±3.96	12.81±1.72	11.54±0.86	11.62±0.53	10.41±2.34	9.32±5.63	10.11±0.86	7.90±3.75
SFA	**31.94±6.72**	**30.05±3.96**	**26.59±2.17**	**28.07±1.46**	**30.12±13.52**	**32.00±11.48**	**29.83±3.85**	**31.78±7.56**
16:1*n*-9	2.14±0.50	2.18±0.32	1.85±0.16	1.96±0.24	1.62±0.30	1.65±0.49	1.79±0.35	1.67±0.15
18:1*n*-9	3.28±1.22	2.43±0.70	2.31±0.53	1.84±0.13	2.67±1.98	1.60±0.67	1.89±0.90	1.89±0.73
18:1*n*-7	2.57±0.59	2.84±0.33	2.58±0.28	2.81±0.42	2.21±0.54	1.70±0.59	2.45±0.55	2.33±0.78
20:1*n*-9/11	4.16±0.33	4.05±0.57	4.36±0.13	3.58±0.24	3.38±0.77	2.70±0.55	3.63±0.52	3.18±0.41
20:1*n*-7	2.63±0.31	2.69±0.32	2.57±0.19	2.36±0.24	2.05±0.55	1.78±0.72	2.17±0.25	2.20±0.46
MUFA	**14.77±2.94**	**14.18±2.24**	**13.66±1.29**	**12.54±1.27**	**11.93±4.15**	**9.43±3.01**	**11.92±2.56**	**11.27±2.53**

Table S5 (cont). Fatty acid profiles (data presented as % of relative abundances) of the adductor muscle of live common cockles *Cerastoderma edule* during their shelf-life at 4 ºC. Values are means of 5 replicates ± standard deviation. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA –polyunsaturated fatty acids and HUFA – highlyunsaturated fatty acids.

Fatty Acid	**Shelf-life time (days)**							
(%)	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
18:2*n*-6	1.45±0.39	1.25±0.19	1.33±0.10	1.25±0.07	1.65±1.56	1.20±0.71	1.19±0.31	1.25±1.05
20:2*n*-9	1.58±0.21	1.53±0.17	1.47±0.25	1.60±0.31	1.32±0.33	1.29±0.50	1.44±0.31	1.50±0.77
22:2*n*-9	4.11±0.50	3.95±0.90	4.94±0.58	3.76±0.33	4.31±1.09	3.65±0.77	4.12±0.80	3.58±0.97
22:2*n*-6	2.37±0.28	2.44±0.70	2.96±0.41	2.31±0.27	2.58±0.70	2.05±0.41	2.53±0.50	1.82±0.58
22:3*n*-6	3.76±1.02	3.45±0.97	4.47±0.56	3.73±0.51	4.67±1.15	4.40±1.40	4.80±0.58	4.11±0.69
PUFA	**13.28±2.40**	**12.63±2.94**	**15.17±1.90**	**12.66±1.48**	**14.53±4.83**	**12.58±3.79**	**14.08±2.50**	**12.26±4.07**
20:4*n*-6	3.81±0.61	3.47±0.44	3.82±0.45	3.19±0.17	3.53±0.87	5.01±4.60	3.32±0.51	5.00±4.64
20:5*n*-3	12.06±1.10	12.94±1.12	12.52±1.35	13.45±0.39	11.72±2.35	9.09±4.35	12.57±1.31	9.37±3.44
22:4*n*-6	1.41±0.24	1.34±0.24	1.54±0.24	1.35±0.33	1.32±0.37	1.24±0.40	1.18±0.15	1.12±0.16
22:4*n*-3	1.19±0.20	1.15±0.11	1.30±0.09	1.16±0.07	1.16±0.27	1.00±0.15	1.11±0.07	0.97±0.05
22:5*n*-3	3.11±0.70	3.33±0.29	3.63±0.39	3.52±0.19	3.10±0.69	3.47±0.62	3.00±0.17	3.32±0.26
22:6*n*-3	16.99±3.60	19.48±2.45	20.66±2.48	22.64±0.34	21.49±3.24	23.89±2.76	22.23±1.15	22.37±1.86
HUFA	**38.57±6.45**	**41.72±4.65**	**43.48±5.01**	**45.31±1.50**	**42.32±7.80**	**43.69±12.87**	**43.42±3.36**	**42.15±10.41**

Appendix E. Supplementary data of section 3.2

SUPPLEMENTARY TABLES

Table S6. Detection limits (DL), percentages of samples above DL and precision estimates (% relative standard deviation, RSD) for the Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) analysis of *Cerastoderma edule* shells. DL are based on blank analyses and are expressed in mmol ratios relative to Ca. External precision estimates are based on % RSD standards certified reference materials (MRC's) for sediments.

Element	DL (mmol^{-1} Ca)	% above DL	% RSD
Mg	0.619	100	3
Sr	1.885	100	3
Ba	0.002	100	3
Mn	0.009	100	2

Table S7. Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) operating conditions

Data acquisition parameters	
Scanning	Peak jump
Dwell time (ms)	10
Reading per replicate	3
Points per spectral peak	1
Sweeps	60
Setup timings	
Uptake	30 s
Washout	60 s
Requirements of argon gas	
Purity	≥ 99,996%
Maximum quantity of water	<5 mg L^{-1}
Flow	14 L min^{-1}
Isotopes measured	^{24}Mg, ^{43}Ca, ^{88}Sr, ^{37}Ba, ^{55}Mn
Internal standard	^{115}In

Table S8. Multivariate analysis of variance (MANOVA) of the trace elements fingerprinting (TEF) of the *Cerastoderma edule* shells between areas within Óbidos lagoon (OL), Tagus estuary (TE), Albufeira lagoon (AL) and Sado estuary (SE).

Area	df	pillai	approx. F	p. value
OL1 vs OL2	1	0.910	38.10	1.08e-07
TE1 vs TE2	1	0.747	11.10	2.19e-04
AL1 vs AL2	1	0.444	3.00	5.25e-02
SE1 vs SE2	1	0.459	2.97	5.71e-02
RF1 vs RF2	1	0.831	18.50	1.15e-05