Universidade de Aveiro Departamento de Biologia

2013

Cláudia Pessoa Dias

Biologia, Ecologia, Dinâmica Populacional e Distribuição de Metais na *Sepia officinalis* numa típica laguna costeira estuarina – Ria de Aveiro

Biology, Ecology, Population Dynamics and Distribution of Metals in *Sepia officinalis* on a typical estuarine coastal lagoon - Ria de Aveiro

Universidade de Aveiro Departamento de Biologia
2013

Cláudia Pessoa Dias

Biologia, Ecologia, Dinâmica Populacional e Distribuição de Metais na *Sepia officinalis* numa típica laguna costeira estuarina – Ria de Aveiro

Biology, Ecology, Population Dynamics and Distribution of Metals in *Sepia officinalis* on a typical estuarine coastal lagoon – Ria de Aveiro

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor António Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro, e co-orientação do Doutor José Pedro Andrade, Professor Catedrático da Faculdade de Ciências do Mar e do Ambiente da Universidade do Algarve.

To my husband, Hugo, for the unconditional support since the beginning until the end of this work.
To my little princesses Francisca, Maria and Catarina.
To them I dedicate this thesis.

o júri

presidente	Prof. Doutor Helmuth Robert Malonek Professor Catedrático da Universidade de Aveiro
vogal	Prof. Doutora Maria João da Anunciação Franco Bebiano Professora Catedrática da Faculdade de Ciências do Mar e do Ambiente da Universidade do Algarve
co-orientador	Prof. Doutor José Pedro Andrade e Silva Andrade Professor Catedrático da Faculdade de Ciências do Mar e do Ambiente da Universidade do Algarve (Coorientador)
vogal	Prof. Doutor Amadeu Mortágua Velho da Maia Soares Professor Catedrático da Universidade de Aveiro
orientador	Prof. Doutor António José Arsénia Nogueira Professor Associado com Agregação da Universidade de Aveiro (Orientador)
vogal	Doutor Carlos Alberto Garcia do Vale Investigador Coordenador, IPIMAR, Unidade de Ambiente Marinho e Biodiversidade de Lisboa
vogal	Doutora Ana Catarina de Almeida Sousa Bolseira pós-Doutoramento do CESAM – Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

agradecimentos

Ao Professor Doutor António Arsénia Nogueira, expresso o meu reconhecimento pelos seus preciosos ensinamentos, pela disponibilidade em me orientar ao longo destes longos anos de trabalho, pela motivação, boa disposição e amizade que constantemente me transmitiu. E ainda pelo forte incentivo para a sua finalização.

Ao Professor Doutor José Pedro Andrade agradeço o apoio científico prestado e pela possibilidade de efetuar este trabalho sob a sua coorientação.

Ao Engenheiro Carlos Vale agradeço o apoio científico prestado, o privilégio de integrar temporariamente a sua equipa de investigação para a realização do trabalho laboratorial referente à quantificação dos metais, as suas sugestões e comentários preciosos.

Ao Instituto Nacional de Investigação Agrária e das Pescas (INIAP-IPIMAR), em especial ao Professor Costa Monteiro, por disponibilizar todos os meios necessários à realização das análises dos metais.

Aos amigos Ramiro Pastorinho e Ana Catarina Sousa agradeço o carinho, a preocupação e a enorme disponibilidade prestada na fase final deste trabalho.

À Joana Raimundo, com quem tive o prazer de partilhar parte do trabalho laboratorial e tratamento dos dados, sempre com a maior dedicação e boa disposição.

Ao Departamento de Ambiente Aquático, em especial ao Vasco Branco, à Rute Cesário, ao Nuno Fonseca e à Patrícia Pereira.

Ao Sr. Aldiro, técnico do Departamento de Biologia, que ajudou na árdua tarefa do trabalho de campo, tornando-a em momentos divertidos, e cuja amizade, empenho e dedicação levaram a bom “Porto” as amostragens.

Às colegas Lúcia Pombo e Carla Lopes, com quem tive o prazer de compartilhar o trabalho de amostragem e laboratorial.

Ao Professor Doutor João Coimbra e à Professora Doutora Maria Armanda Henriques pelo apoio e forte incentivo ao término deste trabalho.

Ao Professor Doutor Agostinho Antunes agradeço o incentivo e compreensão para a finalização deste trabalho.

À Dra. Fernanda Reis, com quem tenho o prazer de trabalhar, agradeço todo o apoio, incentivo e preocupação demonstrados durante este trabalho.

Aos meus irmãos e sobrinhos pela alegria contagiante de todos, pela preocupação e pelo carinho demonstrados ao longo destes anos.

Às minhas irmãs Raquel, Sandra e Cristina expresso a minha gratidão pelo precioso apoio na parte final deste trabalho. Obrigada manas!

À minha Avó, a quem muito devo!

Aos meus pais, a grande referência da minha vida! Obrigada por tudo e por serem quem são!

palavras-chave Choco, ciclo de vida, maturação sexual, metais, bioacumulação, glândula digestiva, Ria de Aveiro

resumo

O choco comum, *Sepia officinalis,* é um cefalópode necto-bentónico que pode viver em ecossistemas de águas costeiras, com elevada influência de pressões antropogénicas e assim, estar vulnerável à exposição por vários contaminantes. O choco é uma espécie de grande importância para a economia local de Aveiro, considerando os dados globais de capturas desta espécie para a Ria de Aveiro. No entanto, os estudos acerca desta espécie na Ria de Aveiro são escassos, desta forma o presente estudo pretende preencher esta lacuna de informação sobre o choco na Ria de Aveiro. O choco entra para a Ria na primavera e no verão para reprodução, e volta para águas mais profundas no inverno. Em termos de abundância, as zonas, este e central da ria, a mais próxima da embocadura com o mar, apresentaram os valores de abundância mais elevados, sendo as regiões mais a norte e a sul dos principais canais, as de abundância mais baixa. Este fato pode estar relacionado com fatores abióticos, como a profundidade, a salinidade e a temperatura. No ponto mais a sul da Ria de Aveiro (Areão) nunca foram capturados chocos, tendo este local apresentado os valores mais baixos de salinidade e profundidade.

O choco apresenta um crescimento alométrico, sendo as fêmeas mais pesadas que os machos, para comprimentos do manto superiores a 82.4 mm. Os machos atingem a maturação sexual primeiro que as fêmeas. Na Ria de Aveiro apenas uma geração de progenitores foi encontrada. O choco apresenta-se como predador oportunista, consumindo uma grande diversidade de presas de diferentes grupos taxonómicos. A dieta mostrou-se semelhante nos diferentes locais de amostragem observando-se diferenças significativas para as estações do ano.

S. officinalis foi capturada em 10 locais da Ria de Aveiro com contaminação de diferentes origens antropogénicas. Assim, os níveis de metais analisados apresentaram-se semelhantes em todos os locais de amostragem, com a exceção de uma área restricta, o largo do Laranjo, que apresentou valores mais elevados. O choco possui a capacidade de acumular metais no seu organismo. Os níveis de Fe, Zn, Cu, Cd, Pb and Hg encontrados no manto e na glândula digestiva refletem uma acumulação diferencial de metais nos tecidos. Esta acumulação está relacionada com o tipo e função do tecido analisado e com o tipo de metal analisado (essencial e não essencial). As concentrações dos metais na glândula digestiva são mais elevadas que no manto, com exceção do mercúrio. Este fato pode dever-se à grande afinidade do manto para a incorporação de Metilmercúrio (MeHg), a mais abundante forma de mercúrio. A acumulação de metais pode variar ao longo da vida, dependendo do metal. As concentrações de Zn, Cd and Hg aumentam ao longo da vida, o Pb diminui e os metais essenciais como o Fe e Cu permanecem constantes. Os dados recolhidos sugerem que o choco (*Sepia officinalis*) possa ser usado como bioindicador de contaminação ambiental para alguns metais.

keywords Cuttlefish, life Cycle, sexual maturity, metals, bioacumulation, digestive gland, Ria de Aveiro

abstract The common cuttlefish, *Sepia officinalis*, is a necto-benthic cephalopod that can live in coastal ecosystems, with high influence of anthropogenic pressures and thus be vulnerable to exposure to various types of contaminants. The cuttlefish is a species of great importance to the local economy of Aveiro, considering the global data of catches of this species in the Ria de Aveiro. However, studies on this species in Ria de Aveiro are scarce, so the present study aims to fill this information gap about the cuttlefish in the Ria de Aveiro. The cuttlefish enters Ria de Aveiro in the spring and summer to reproduce, returning to deeper waters in the winter. In terms of abundance, the eastern and center regions of the lagoon, closer to the sea, showed the highest values of abundance, while the northern and southern regions of the main channel had the lowest abundance. This fact may be related to abiotic factors, as well as depth, salinity and temperature. In the most southern point of the Ria de Aveiro (Areão) no cuttlefish was caught. This site had the lowest values of salinity and depth.

The cuttlefish has an allometric the females being heavier than males to mantle lengths greater than 82.4 mm. Males reach sexual maturity first than females. In Ria de Aveiro in a generation of parents was found. The cuttlefish, presents itself as opportunistic predators, consuming a wide variety of prey from different taxa. The diet was similar in different sampling locations observing significant differences for the seasons.

S. officinalis was captured at 10 sites in the Ria de Aveiro with different anthropogenic sources of contamination. Thus, levels of metals analyzed were similar at all sampling sites, with the exception of a restricted area, Laranjo, which showed higher values. The cuttlefish has the ability to accumulate metals in your body. The levels of Fe, Zn, Cu, Cd, Pb and Hg found in the digestive gland and mantle reflect a differential accumulation of metals in the tissues. This accumulation is related to the type and function of tissue analyzed and the type of metal analysis (essential and non-essential). The metal concentrations in the digestive gland are higher than in the mantle, with the exception of mercury. This may be due to the high affinity of the mantle for the incorporation of methylmercury (MeHg), the most abundant form of mercury. The accumulation of metals can vary over a lifetime, depending on the metal. The concentrations of Zn, Cd and Hg increases throughout life, while Pb decreases and essential metals such as Fe and Cu remain constant. The data collected suggest that the cuttlefish (*Sepia officinalis*) can be used as a bioindicator of environmental contamination for some metals.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABREVIATIONS

AFDW	Ash-free dry weight;
Ag	Silver;
ANCOVA	Statistical software;
ANOVA	Factorial analysis of variance;
APHA	American public health association;
ARE	Areão sample site;
As	Arsenium;
BAR	Barra sample site;
bt	Bottom trawl;
CANOCO	Statistical software;
CAR	Carregal sample site;
Co	Cobalt;
Cr	Chromium;
Cu	Copper;
DGI	Digestive gland index;
DGPA	General Management of Fisheries and Aquaculture;
DML	Dorsal mantle length;
DOLT	International certificate standards fish liver;
DORM	International certificate standards dogfish muscle;
Eh	Redox potential;
EPA	Environmental Protection Agency;
F	Fluorine; statistical parameter (ANOVA);
Fe	Iron;
GAF	Gafanha sample site;
GSI	Gonadosomatic index;
GW	Wet weight of the gonad;
$\mathbf{H_2O_2}$	Hydrogen peroxide;
Hg	Mercury;
HMW	High-molecular weight ligands;
$\mathbf{HNO_3}$	Nitric acid;
HSP	Heat shock proteins;
I	Iodium;

ICP-MS	Inductively couple plasma mass spectrometry;
INE	National Institute of Statistics;
INIAP	National Institute for Agricultural and Fisheries Research;
IPIMAR	National Institute of Biological Resources (now IPMA – Portuguese Institute of Ocean and Atmosphere);
K	Potassium;
LAR	Laranjo sample site;
LMW	Low-molecular-weight ligands;
MeHg	Methylmercury;
MINITAB	Statistical software;
ML	Mantle length;
Mn	Manganese;
MRG	Metal-rich granules;
MTs	Metallothioneins;
MTLP	Metallothioneins-like proteins;
Ni	Nickel;
NOOA	National Oceanic and Atmospheric Administration;
NS	Not significant;
nt	Network trawl;
OSPAR	Convention for the Protection of the Marine Environment of the North-East Atlantic;
p	Probability;
Pb	Lead;
PCA	Principal Component Analysis;
pH	measure of the acidity or basicity of an aqueous solution;
PM	Percentage of sexually mature individuals;
POR	Commercial port;
RDA	Redundancy analysis;
RIO	Rio novo do príncipe sample site;
S	Significant;
SD	Standard deviation;
SH-groups	Sulphur groups;
Se	Selenium;
SFM	Size at first maturation;

SJA	S. Jacinto sample site;
Sn	Tin;
TOR	Torreira sample site;
TORT	International certificate standards lobster hepatopancreas;
UNEP	United Nations Environment Programme;
V	Vanadium;
VAG	Vagos sample site;
WFD	European Water Framework Directive;
Wt	Total weight;
Zn	Zinc;
WWt	Wet weight;
DWt	Dry weight;
GLM	General Linear module for ANOVA.

CHAPTER I

General Introduction

I. General Introduction

I.1. Coastal Environment – An overview

Coastal environments are dynamic ecosystems where land, water and atmosphere interact in a delicate natural balance that is constantly affected by natural and human influence (Lopes *et al*., 2011). Consequently these ecosystems are easily susceptible to detrimental environmental impact from pollution or by other human activities (Pombo, 2005). Natural pressures include storms, wind, tides, waves, sea level change and runoff (Lopes *et al*., 2011), while anthropogenic pressures include random urbanization and industrialization, unregulated wastewater discharges (domestic and industrial waste) and reduction of resources (overfishing and unplanned coastal agriculture) (Rabouille *et al.*, 2007). Coastal lagoons belong to these important ecosystems and are among the most productive ecosystems of the biosphere, where several organisms use the lagoon for nesting, feeding, nursery or sheltering (McLuscky and Elliott, 2004; Sousa *et al*., 2010). These productive areas are attractive and accessible to people and therefore densely populated, making the anthropogenic pressures an important factor in their evolution (Lopes *et al*., 2011). Aproximately 44 % of the world's population (more people than inhabited the entire globe in 1950) live within 150 km of the coast. In 2001 over half the world's population lived within 200 km of a coastline. Moreover, increasing volumes of waste, particularly sewage, are discharged into coastal waters with little or no treatment, what can cause eutrophication and endanger public health (UN Atlas of the Oceans, 2010). Concerns of adverse long-term contaminant effects on aquatic ecosystems emerged in the last decades (Van der Oost *et al.*, 2003). The protection, improvement and sustainable use of Europe´s water resources are the main goals of current European Water Policy (Kowalshi, 2009). In order to achieve this aim, legislation is defined by the European Union Water Framework Directive (WFD). The great objective is to achieve good quality water status for all European aquatic ecosystems until 2015. However, the complexity and dynamics of these coastal environments and the constant change of these ecosystems due to natural causes may overlap the effects of the anthropogenic pressures, hampering the monitoring and the management of these important aquatic ecosystems.

I.2. Tools for monitoring aquatic systems

Metal pollution has been an environmental issue in many developed and developing countries for decades, and there is a substantial need to understand the bioaccumulation and toxicity of metals in aquatic organisms (Wang and Rainbow, 2008). Over the last two or three decades, researchers proposed the use of living organisms in parallel with physical-chemical analyses to determine geographical and/or temporal variations in the bioavailable concentrations of trace metals, in order to evaluate the health state of the aquatic systems (Amiard *et al*., 1998; Rainbow and Phillips, 1993). Considering this, biomonitoring was defined as the systematic use of biological responses to evaluate changes in the environment (Cairns and Van der Schalie, 1980). This approach is based on the knowledge that chemicals which have entered the organisms leave markers reflecting that exposure. The marker may be the chemical itself, a breakdown product or a biological change in the organism as a result of the action of the chemical (Kowalshi, 2009).

I.2.1. Bioindicator, biomonitors or sentinel species

Bioindicators, biomonitors or sentinel species can be defined as species which accumulate contaminants in their tissues (Beeby, 2001). Their use to identify regions with high concentrations of trace metals and synthetic organic concentrations in aquatic ecosystems is particularly well established, especially in temperate regions. Bioindicators can provide a time-integrated measure of bioavailability, responding, by definition, to the fraction present in the environment which is of direct ecotoxicological relevance (Phillips and Rainbow, 1993). Individual bioindicators respond differently to different sources of bioavailable metal, for example, in solution, in sediment or in food. To gain a complete representation of total trace metal bioavailability in a marine system it is necessary, therefore, to use a set of bioindicators species, reflecting metal bioavailability in all available sources (Phillips, 1990; Rainbow, 1990, 1993; Phillips and Rainbow 1993; Rainbow and Phillips, 1993; Galloway *et al.*, 2004). Such comparative use of different biomonitors should allow identification of the particular source of the contaminant metal (Phillips and Rainbow, 1988, 1993). For example, a macrophytic algae responds essentially to dissolved metal sources, a suspension feeder like a mussel responds to metal sources in dissolved and suspended phases, and a deposit feeder responds to metal available in the sediment.

Trace metals bioindicators need to conform to certain required characteristics, not least being metal accumulators (Butler *et al.,* 1971; Bryan *et al.,* 1980; Phillips, 1990; Phillips and Rainbow, 1993). Ideal bioindicators should be sedentary, easy to identify, abundant, long lived, available for sampling throughout the year, large enough to provide sufficient tissue for (individual) analysis, resistant to handling stress caused by laboratory studies of metal kinetics and/or field transplantations, tolerant of exposure to environmental variations in physical-chemical parameters such as salinity, and net accumulators of the metal in question with a simple correlation between metal concentration in tissues (body) and average ambient bioavailable metal concentration over a recent time period (Rainbow, 1995). However, not all bioindicators organisms follow all these criteria. Bivalves, particularly mussels, are one of the most relevant sentinel species (NOAA, 2004), as they are used in international environmental monitoring programs of marine contaminants as part of “Mussel Watch Programme” of the National Oceanic and Atmospheric Administration (Goldeberg *et al.* 1983). Fishes are good contamination indicators but despite having a considerable commercial value, they have great mobility. Along with fish, plankton is also used as bioindicator but strongly depend of the currents and local conditions and their sampling is often difficult. Molluscs, and especially mussels, are the most used organisms to monitor trace metals in coastal environments due to their abundance, physiology and distribution (Pimenta, 2010).

Bioindicators species in biomonitoring programs can have different uses (Beeby, 2001):

a) As accumulators, when used to increase analytical sensitivity for a contaminant; to compare the scale of contamination between sites, and to summarize a complex pollution signal;

b) As integrators, when used to provide a running mean over time and space;

c) As a measure of exposure, when used to quantify bioavailability of a pollutant from a particular source.

I.2.2. Biomarkers

A biological marker or biomarker is defined as quantifiable behavioural, physiological, histological, biochemical or genetic property that is used to measure response to an environmental change (Nordberg *et al.*, 2009). The use of biomarkers measured at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurement in environmental quality assessment (McCarthy and Shugart, 1990). Newly the use of biomarker approach has been incorporated into several pollution monitoring programmes in Europe and the USA (e.g. the NOAA's National Status and Trends Program). The United Nations Environment Programme has funded a biomonitoring programme in the Mediterranean Sea including a variety of biomarkers (UNEP, 1997). Recently, biomarkers have also been included in the Joint Monitoring Programme of the OSPAR convention where Portugal and Spain are members (Cajaraville *et al.*, 2000).

Considering the definitions of the World Health Organisation (1993), biomarkers can be classified as:

a) biomarkers of exposure: an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;
b) biomarkers of effect: a measurable biochemical, physiological, behavioral or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease;
c) Biomarkers of susceptibility: an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance.

One of the most important features of molecular/cellular biomarkers is that they have the potential to anticipate changes at higher levels of biological organisation, i.e. population, community or ecosystem. Thus these 'early warning' biomarkers can be used in a predictive way, allowing the initiation of bioremediation strategies before irreversible environmental damage of ecological consequences occurs. Biomarkers are then defined as short-term indicators of long-term biological effects (Cajaraville *et al.*, 2000).

I.3. Metals in coastal environments

An increasing variety of industrial and agricultural chemicals are introduced in coastal ecosystems. Metals are of great environmental concern, since they tend to concentrate in marine organisms, are virtually non-degradable, and thus produce long lasting effects upon the environment even after their major sources have been removed (Kowalshi, 2009).

Trace metals are defined as elements that occur in trace amounts within the environment or within organisms (typically <0.01% of the organism (Wittman, 1979)). The Class A of macronutrients (Na^{+}, K^{+}, Ca^{2+} and Mg^{2+}) are excluded from these group, but the class B of ions are included, readily binding with sulphur, Cu^{+}, Ag^{+}, Hg^{2+} and Pb (IV), and ions with an intermediate sulphur binding ability, Mn^{2+}, Zn^{2+}, Cr^{2+}, Fe^{3+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Sn^{2+}, Sn (IV), Pb^{2+} and As (III) (Nieboer and Richardson, 1980; Rainbow, 1997b) Metals are classified as essential, when their biological roles are well-known, such occurs with Fe, Cu, Zn, I, Mn, Se or F and non-essential, when their physiological functions have not been clearly demonstrated, such as occurs with Ni, V; Ag, Hg, Pb and Cd (Lall, 1995; Bustamante, 1998). Essential metals always function in combination with organic molecules, usually proteins (Blackmore, 1998). For example, Cu, Zn and Fe are important components of the catalytic sites of enzymes, respiratory proteins and certain structural elements of the organisms (Depledge and Rainbow, 1990). Other metals like Pb, Hg and Cd may relocate or substitute essential metals and interfere with the good functioning of the enzymes and associated cofactors (Van der Oost *et al.*, 2003). As mentioned previously some trace metals are essential in low concentrations for the metabolism of organisms, but in excess all trace metals are toxic. In the environment, metals occur in a variety of forms or species affecting their toxicity (McLusky *et al.*, 1986; Rainbow, 1997a). Besides, the toxicity of metal mixtures often cannot be predicted based on the toxicity of individual metals (Chapman *et al.*, 2003). In addition, some of these elements appear in high concentrations in certain organs of the aquatic organisms, and the question is if it results from biological processes or influenced by the increasing availability in contaminant environments.

There are three fates for trace metals in aquatic ecosystems: a) precipitation, which occurs when the concentration of the metal is higher than its solubility and where changes in solubility will occur with, for example, pH or when freshwater meets seawater in an estuary; b) adsorption, onto both inorganic and organic matter; and c) absorption by marine organisms (Bryan, 1979). The inputs of metals into marine ecosystems have several causes like effluent discharges, fluvial currents and by the atmosphere. Once in the water, metals adsorbed on the suspended particles and then accumulate in the sediment (Ramalhosa *et al.*, 2001; Ramalhosa *et al.*, 2006), which can serve as sink or source of metals to the overlying water column (Fichet *et al.*, 1999; Baeyers *et al.*, 2003). Marine and estuarine sediments bind and accumulate a wide variety of trace metals to often high concentrations, and can be redistributed by disturbances (including bioturbation by animals), and anthropogenic activities such as dredging (Chapman *et al*., 2003). Several marine organisms live a substantial part of their life in or on the sediment, which enhances the possibility to incorporate those metals. The sediment-water transferences of metals are controlled by two types of flux. The advective flux, when the transference of metals between the sediment and the water column is conducted by an external factor, such as tidal currents or bioturbation. And, the diffuse flux where the natural diffusion of metals occurs between the two phases, with a direction and rang depending on the depleted/enriched section (Point *et al*., 2007). The mineralization of organic matter plays an important role in the sediment-water interactions (Kowalshi, 2009). Iron and Mn oxides in the sediment are important scavengers for metals. However, during this process, ions Mn^{2+} and Fe^{2+} and associated trace metals (Cu, Pb, Zn and Cd) can be released into the pore-water and diffuse upward due to concentration gradients. According to Miao *et al.*, 2006 this process is largely affected by Eh and pH.

I.4. Sequestration of metals in aquatic organisms

Sequestration of metals in organisms occurs throughout the various tissues involved in pathways for metal uptake, storage, elimination and detoxification (Figure I.1).

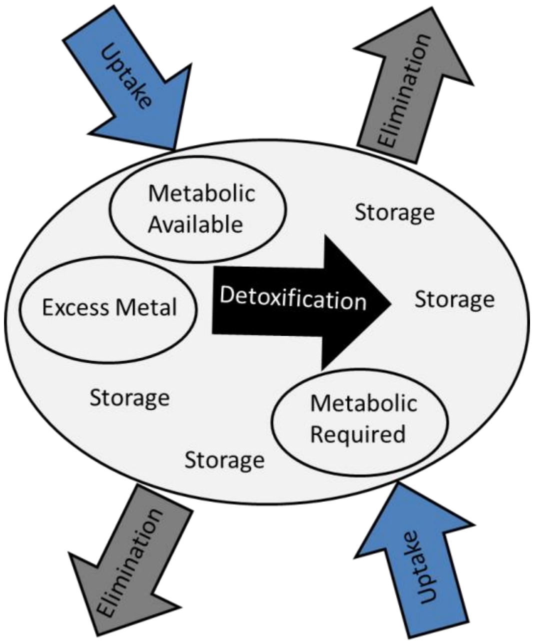

Figure I.1 - Sequestration of metals (uptake, storage, elimination and detoxification (Adapted from Pimenta, 2010).

I.4.1. Uptake of metals

The uptake of metals in aquatic organisms can occur through different mechanisms: from water, suspended particles and sediments, or by food. The accumulation of trace elements directly by water is a crucial point to consider in the evaluation of the adverse effects on ecosystems (Van der Oost *et al.*, 2003). Indeed, there are many works that use the accumulation of trace elements in organisms as mean to evaluate the environmental health condition (Fernandes *et al*., 2007; Bustamante *et al.*, 2008; Pereira *et al.*, 2009). The internal transport of metal is made via circulatory system (Roesijadi and Robinson, 1994). The pathways of trace metal transport across the biological membrane have been well hypothesized, such as the passive diffusion of neutral metal species across the membrane, facilitated diffusion of metals via membrane transporter proteins, access through major ion channels (e.g., Ca channels) perhaps in association with active ion pumps, and endocytosis (Simkiss and Taylor, 1995; Hudson, 1998). Pumps and channels are in the membranes, and have hydrophobic segments that bind metals. The carriers are liposoluble molecules which bind to metals in one side of membranes, make the passage and transport the metal to the solution on the opposite side of the membrane (Hudson, 1998). Dissolved metals are mainly taken up by exposed body surfaces such as the gills, whereas particulate metals are mostly ingested and then taken up after solubilisation in the gut. Uptake of essential metals (e.g. Cu, Fe and Zn), often involves specific pathways like, calcium channels and specific membrane carriers for Fe and Cu. For common divalent metals such as Cd and Zn, there are two different types of binding sites at the gill surface, a high-affinity, low-capacity site, and a low-affinity, high-capacity site. At a relatively low ambient metal concentration, metals are bound to the high-affinity, low-capacity site, and uptake exhibits a saturation pattern with increasing ambient metal concentrations. At a high ambient metal concentration, following the saturation of the high-affinity sites, metals are bound to the low-affinity, high-capacity site, and uptake increases linearly with increasing ambient metal concentration (Wang and Rainbow, 2008). For non-essential elements such as Hg, specific uptake mechanisms are unknown and so, it’s believed that they should follow the existing pathways for essential metals (Hudson, 1998, Sunda and Huntsman, 1998). Uptake of non-essential metals is almost totally determined by the degree of exposure. In contrast, accumulation of some essential metals may be less influenced by external concentrations, suggesting various degrees of homeostasis (Langston *et al.* 1998). In addition, numerous environmental and biological factors including dissolved metal interactions (Amiard-Triquet and Amiard, 1998) have been considered for their influence on trace metal uptake and bioaccumulation.

In organisms without external shells, as cephalopods, the uptake from water can take place in the gills but also in all body surface, and in all molluscs some absorption of dissolved metal across the digestive epithelium (Langston *et al.*, 1998). Food may also be a significant source of metals, if not the primary source, to organisms (Wang, 2002). For marine organisms dietary uptake (trophic transfer) is the predominant pathway by which metals are accumulated, primarily because of the very low dissolved uptake rate of metals (Xu and Wang, 2002; Zhang and Wang, 2006a, b).

I.4.2. Bioaccumulation

Bioaccumulation in a tissue can be considered as a balance between two kinetic processes accumulation and elimination of an element (Connel and Miller, 1984, Brown and Depledge, 1998). Generally, the accumulation of metals occurs in a pattern described in the Figure I.2. When an organism is exposed to a metal, levels increase logarithmically until adsorption sites become full (Langston *et al.*, 1998), leading to a reduction in the accumulation. The elimination process becomes clear when a decrease in metal levels in the tissues of the organisms is observed. However, not all the metals are rapidly eliminated, depending on its half-life. Metal exposure may induce specific metal detoxification processes or physiological and biochemical changes that can subsequently affect the uptake of metals (Wang and Rainbow, 2005). Over the last few decades, numerous studies have been carried out on the uptake and bioaccumulation of trace metals by aquatic animals (Hamelink *et al.*, 1994; Turner and Tessier, 1995). The bioaccumulation of trace metals depends on the physiological accumulation pattern of the species for each metal. If organisms with accumulation patterns with increasing dependence on storage accumulation follow sequentially along a food chain, the effect will mimic biomagnification of trace metals along food chains (Amiard-Triquet *et al.*, 1993; Wang, 2002).

The different bioaccumulation strategies go from a high accumulation and weak depuration to weak accumulation and a high depuration. The subsequent fate of the element depends on the particular physiology of the organism, as to whether the metal is used for an essential metabolic purpose, stored in the body, excreted, or even gains access to the “wrong” biomolecule (Rainbow, 2002).

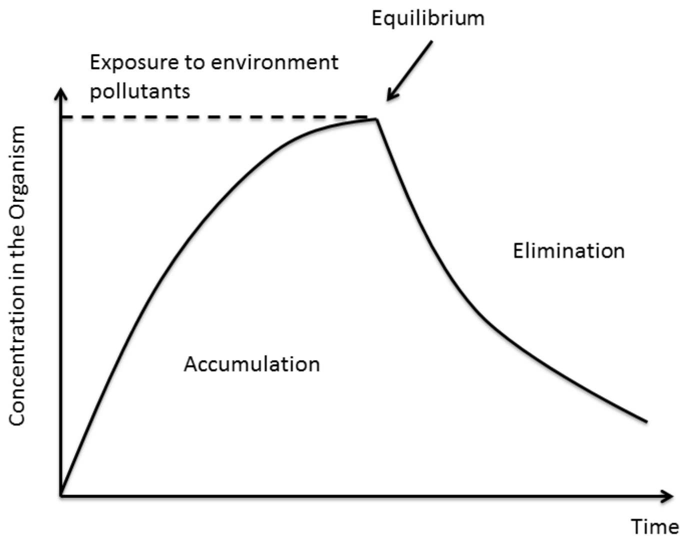

Figure I.2 – Accumulation and elimination of metals by organisms (Adapted from Connel and Miller, 1984).

I.4.3. Detoxification

Aquatic organisms have the ability to accumulate high levels of essential and non-essential metals, and therefore they need to develop effective detoxification systems (Finger and Smith, 1987; Bebianno and Serafim, 1998). Once exposure has occurred and substances are bioavailable, a sequence of biological responses may take place. Intrinsic (e.g. sex, age, health and nutritional status) and extrinsic (e.g. duration, dose, route of exposure to the contaminant and the presence of other chemicals) factors may influence the health of the organisms even when they are exposed to trace elements at undesirable concentrations (Van der Oost *et al.*, 2003). Trace metals in excess are potentially toxic and should be removed from the vicinity of important biological molecules to maintain the regular function of cells. Subcellular metal partitioning is the basis of internal metal sequestration over different organs and tissues, depending upon many factors such as metal type and metal pre-exposure (Vijver *et al.*, 2004). Indeed, it is generally accepted that toxicity will manifest itself at the subcellular level before it is observed at higher levels of biological organisation (Cajaraville *et al.*, 2000).

Aquatic organisms have evolved a number of subcellular systems for accumulation, regulation and detoxification of the excess of essential and non-essential metals, which allowed aquatic organisms to survive within environments containing toxic metals (Bebianno, 1990; Bebianno and Serafim, 1998; Isani *et al.*, 2000).

Currently, metals are generally fractionated into 5 operationally defined subcellular pools, consisting of metal-rich granules (MRG), cellular debris (mainly cellular membrane fragments), organelles (metals bound with mitochondria, lysosomes, endoplasmic reticulum), heat shock proteins (HSP, including enzymes), high-molecular-weight ligands (HMW, such as metalloenzymes), low-molecular-weight ligands (LMW, e.g. glutathione) and heat-resistant proteins (generally considered to be metallothioneins (MTs) or more correctly metallothionein-like proteins MTLP) (Wallace *et al*., 2003). Overall, systems at subcellular level may be activated in order to prevent harmful effects. The onset of toxicity can happen at any total body concentration if the uptake rate changes such that it exceeds the combined rates of excretion and detoxification for sufficient time for the concentration of metabolically available metal to exceed a threshold (Rainbow, 2007).

One of the best studied subcellular structures are the MTs, low-molecular-weight-rich-proteins, metal-binding protein rich in sulphur groups, with high affinity for group B metal ions (e.g. Zn, Cu, Cd and Hg) (OSPAR commission, 2013). MTs are found in all vertebrates and most invertebrates, being identified in approximately 50 different species of aquatic invertebrates, the majority of which are molluscs or crustaceans.

Most forms of MTs are involved in metal-sequestration, thereby possibly: regulating cellular processes requiring Zn and/or Cu; and binding and thus temporarily detoxifying non-essential elements such as Cd and Hg. In addition, MT has been suggested to be involved in the cellular defense against free radicals (mainly due to the large number of SH-groups) (OSPAR commission, 2013). Consequently, they have become of great interest for assessing pollution in the marine environment and are seen as potential biomarkers of metal exposure in molluscs and fish (Langston *et al*., 1998). Trace metals can induce MTs (Bebiano *et al.*, 1993; Bebianno and Serafim, 1998; Lueng and Furness, 2001; Shi and Wang, 2005), which are involved in cellular regulation (homeostasis) and detoxification of these metals (Bebiano and Langston, 1998; Roesijadi *et al.*, 1998; Viarengo *et al.*, 1998; Wang and Rainbow, 2005; Amiard *et al*., 2006). In marine fish species, MT concentration in tissues has been found to be most strongly associated with Zn and Cu levels, although Cd may also result in minor increases in areas with metal stress (Hylland *et al*., 2006). The fact that aquatic organisms have the capacity to synthesize these MTs that can sequester and subsequently detoxify metals implies that the increased body burden of metals will not necessarily result in increased toxic effects (Di Giulio *et al*., 1995).

Aquatic organisms utilize a variety of mechanism to eliminate metals. The kinetic of metal release is complex and reflects the diverse compartments from which metals must be mobilized. Additionally, physical and chemical parameters, such as temperature and salinity, may affect the rate of release in aquatic organisms, which can use several pathways to release metals: renal, digestive or diapedesis (in molluscs) (Kowalshi, 2009). Salinity is one of the environmental factors that can have a wide variety of effects on the chemical speciation of river-borne trace metals such as desorption of particle-bound metals, organic complexation and removal of trace metals from solution (Li *et al.*, 1984).

I.5. General characterization of *Sepia officinalis* (common cuttlefish)

I.5.1. Taxonomic position

According to Sweeney and Roper (1998), the taxonomic position of *Sepia officinalis* (Figure I.3) is:

Phylum MOLLUSCA Linnaeus, 1758
Class CEPHALOPODA Cuvier, 1797
Subclass COLEOIDEA Bather, 1888
Order SEPIIDA Zittel, 1895
Family SEPIIDAE Leach, 1817
Genus *Sepia* Linnaeus, 1758
Specie *Sepia officinalis* Linnaeus, 1758

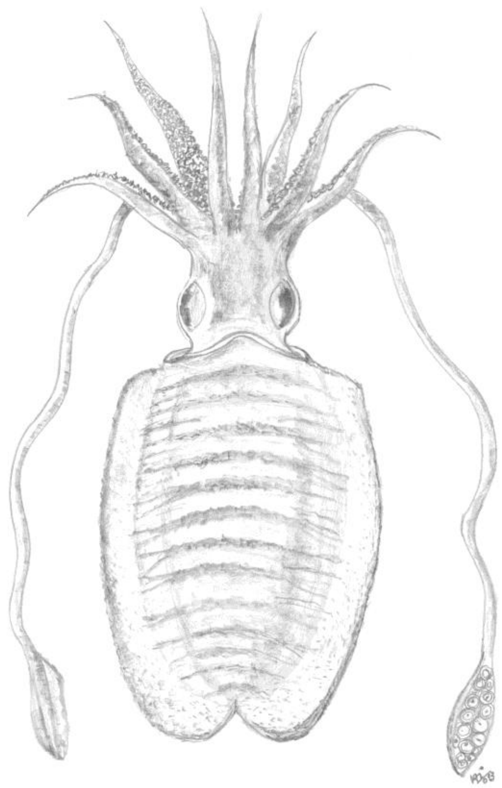

Figure I.3 – Dorsal view of *Sepia officinalis*.

Common names: Choco (Portugal), Cuttlefish (UK, USA), Rellema or Choco (Spain), Sepia or Seccia (Italy) and Seiche (France).

I.5.2. World distribution

The common cuttlefish, *Sepia officinalis* Linnaeus, 1758, is one of the world's best known cephalopods. The specie presents a wide distribution (Figure I.4), is found in eastern Atlantic shelf waters (Nixon and Mangold, 1998), from southern Norway to south Mediterranean sea (Roper *et al.*, 1984), and in the north Africa until Cap Blanc (Boletzky, 1983; Kromov *et al.*, 1998). Further south in African coast *Sepia officinalis* is replaced by the subspecie *Sepia officinalis hierreda* (Guerra, 1987; Guerra *et al.*, 2001). Common cuttlefish is absent from American coast (Roper *et al.*, 1984; Guerra, 1987; Nash and Thorpe, 2003). In Portugal, cuttlefish is common among all Portuguese coast (Roper *et al.*, 1984), however it is more abundant on west coast, at south of the Tagus river and in Algarve Coast (south Portugal) (Brito, 1998). There are approximately 100 species in the genus *Sepia*, in Europe’s coastal waters (Roper *et al.*, 1984; Pierce *et al.*, 2010). In the same area of distribution also exists, *Sepia elegans* and *Sepia orbignyana*, species from the same genus of *Sepia officinalis* (Guerra, 1987; Pierce *et al.*, 2010). Within these areas the specie lives in coastal waters and on continental shelf (Boletzky, 1983) (Figure I.4). These species of the continental shelf comprise only about 15% of all the cephalopods and live in waters with less than 300 m depth, which covers 6% of the Earth’s surface (Clarke, 1996).

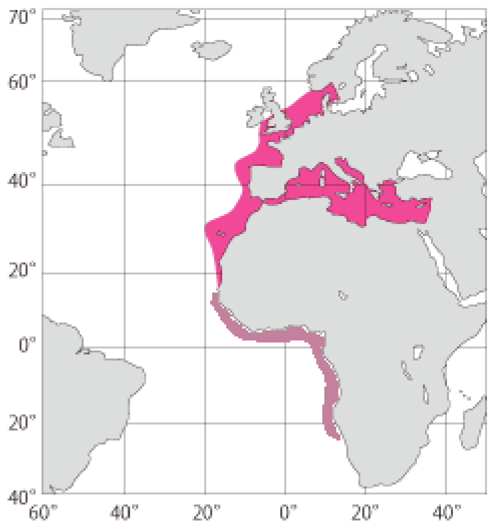

Figure I.4 - Geographical distribution of *Sepia officinalis* (in pink) and *Sepia officinalis hierredda* (in violet) (Adapted from Okutani, 2005).

I.5.3. Morphological characteristics

The diagnostic characteristics of *Sepia* sp. genus are: size; number and arrangement of suckers in the tentacle club, as well as the sepion (cuttlebone) shape (Roper *et al.,* 1984). According to Guerra (1992), the specie *Sepia officinalis* is distinct from others of the same genus by the following features:

a) Body is broad and flattened, and is therefore oval in cross-section;
b) Shape of the internal, porous and calcareous shell know as cuttlebone or sepion;
c) Projection of the mantle over the head forming an obtuse angle;
d) Encircling the mouth there are ten appendices: eight arms with suckers, used to manipulate prey, and two tentacles with flattened paddle-like tips, used to catch prey;
e) tentacles are retractable into bags located on both sides of the head;
f) Arms are short and possess 4 rows of suckers, with chitinous rings while tentacular clubs possess 5-6 suckers in transverse rows, where the median suckers are moderately enlarged;
g) In males the left arm IV is hectocotylized where the normal-sized suckers are replaced by a series of minute suckers scattered over a large area of transverse folds, the hectocotylus.

Mantle of common cuttlefish reaches a maximum length of 45 cm. In terms of weight, cuttlefish can reach up to 4 kg in temperate waters, not reaching more than 30 cm, and 2 Kg in subtropical waters (Ropert *et al*., 1984*).*

Cuttlefish are one of few animal groups with the ability to camouflage themselves on a wide variety of backgrounds, from open sandy plains to complex coral and rock reef habitats (Barbosa *et al*., 2008; Hanlon and Messenger, 1988). Camouflage is the primary defence in cuttlefish (Chiao *et al.,* 2009).

I.5.4. Ecology

Sepia officinalis is a necto-benthic specie occurring predominantly on sandy and muddy bottoms from the intertidal zone to approximately 200m depth, with the highest abundance in the upper 100 m (OSPAR, 2000; Norman, 2000). Although excellent swimmers, they generally are bottom dwellers (Roper *et al*., 1984).

Cuttlefish can live in a range of salinity between 20 and 38 (Guerra and Castro, 1988). In aquaculture, both embryonic development and growth of young cuttlefish, usually occurs between 36 to 37 (Domingues *et al*., 2001a; Domingues *et al.,* 2004a; Baeza-Rojano *et al*., 2010). The range of temperature tolerated by cuttlefish is 10ºC to 30ºC. At temperatures below 10ºC the individuals do not feed, stay inactive and die after a couple of days (Richard, 1971; Bettencourt, 2000). Due to its capacity to adapt to a large range of these external parameters (temperature and salinity), the occurrence of this species is frequent in coastal lagoons, where cuttlefishs are exposed to unstable hydrological conditions, along the year (Palmegiano and D'Apote, 1983; Guerra *et al*., 1988; Serrano, 1992; Guerra, 2006).

The calcified shell of *S. officinalis* is internal and assists the animal in locomotion by facilitating buoyancy (Bassaglia *et al*., 2013). Experiments with cuttlefish showed that cuttlebone of large animals implode between 150 m to 200 m, whereas advanced embryonic specimens and newly hatched implode between 50 m to 100 m. The larger individuals are occasionally caught at depths greater than the implosion depth of juveniles shell parts. They apparently avoid the implosion of the early shell portions by refilling these first-formed chambers with cameral liquid later in life (Ward and Boletzky, 1984).

Captive-raised experiments showed that behaviour of cuttlefish was intensely affected by housing conditions and suggested that this specie is probably semi-solitary under natural conditions (Boal *et al.,* 1999). Domingues *et al*. (2003) revealed that *S. officinalis* cultured in isolation conditions had higher growth and survival rates than the ones maintained at relatively high densities, even when stressed. The authors observed agonistic behaviour related with competition for space with higher densities in tanks, indicating that density, or lack of space, appear to be more limiting than isolation.

I.5.5. Reproductive traits

Fertility in *Sepia officinalis* varies typically between 200 and 3000 eggs per female (Boletzky, 1988). Sexual maturation is conditioned by temperature and light. High temperatures improve the sexual maturation and light acts in the optical glandular system (weak light accelerates the process). Males reach sexual maturity earlier than females (Richard, 1971; Boletzky, 1983; Guerra and Castro, 1988), and in both genders the sexual maturation does not depend on the size of the individual (Boletzky, 1983).

During reproduction season, males protect the females, and become more aggressive, attacking everything or everyone approaching (Richard, 1971).

Cuttlefish is migratory specie of coastal waters that enters shallow coastal ecosystems for reproduction. Spawning generally takes place in coastal waters in depths rarely above the 30 to 40 m (Boletzky, 1983; Boucaud-Camou and Boismery, 1991). The life cycle and biology of this species are well known in several areas, as northwest Mediterranean (Mangold, 1966), English Channel (Richard, 1971), Sea Catalonia (Mangold-wirz, 1963), Ria de Vigo (Guerra and Castro, 1988) and Ria Formosa (Coelho and Martins, 1989; Martins, 1990). However, since the majority of the studies on *Sepia officinalis* have been based on laboratory-reared individuals under controlled conditions, some aspects of its ecology may remain unknown. Therefore there is the need of extending the knowledge of this specie to other areas of its distributional range, like northwest of Portugal. The main spawning season covers spring and summer, but there is also winter spawning, which is especially pronounced on the Atlantic coast (Pierce *et al.*, 2010).

In its natural environment, eggs are laid near the coast in spring. Particularly in the English Channel and Atlantic Ocean, they may be exposed to air and/or desalinated rainwater due to daily tides. Eggs are attached in batches and surrounded by thick black gelatinous envelopes (capsule) potentially protecting the embryo from environmental aggressions (Bassaglia *et al.*, 2013). The eggs sizes usually range between 8 to 10 mm of diameter and are fixated in the shape of a bunch to algae, shells or other substrates (Boletzky, 1983; Roper *et al.*, 1984). Larvae hatch as tiny replicas of adults assuming also their behaviour, namely the benthonic way of life (Boletzky, 1983).

The mantle length of young individuals varies between 8 and 9 mm (Boletzky, 1979).

Temperature influences the growing rate of individuals, which increases with the temperature, and the same trend is verified with the food availability (Richard, 1971).

Cuttlefish has one of most complex nervous systems of all invertebrates. The connection between the brain cortex and the optical lobes is particularly developed (Boucaud-Camou, 1990).

As several other cephalopod species, *S. officinalis* has an extremely high growth ratio, and his life span does not exceed two years. Females undergo just one reproduction round and die shortly after (Bassaglia *et al.*, 2013). Cephalopods are the unique multicellular species with an exponential growth during a significant part of its life cycle (Monteiro *et al.*, 1992).

I.5.6. Trophic Ecology

In general, cephalopods are active carnivores that feed on living preys during their entire life cycle (Boucher-Rodoni *et al.*, 1987). The diet of *S. officinalis* includes a wide variety of prey, like fishes, crustaceans, molluscs, polychaetes, bivalves, nemertean worms, gastropods (Boucaud-Camou *et al.*, 1985, Castro and Guerra, 1989, Pinczon du Sel, 1992, Pinczon du Sel *et al.*, 2000; Domingues *et al.*, 2004b, Alves, 2006). Species composition within these prey groups depends upon the respective species composition and availability in each ecosystem. Large cuttlefish are also cannibals, capturing and eating smaller individuals (Richard, 1971; Castro and Guerra, 1990; Pinczon du Sel and Daguzan, 1992). Considering the wide variety of preys eaten by cuttlefish, this specie should be considered as trophic opportunistic animal (Alves, 2006). Over age there is a progressive replacement of prey, e.g. crustaceans by fishes (Castro and Guerra, 1990), this ontogenetic changes are also related with prey size (Blanc *et al*, 1999; Blanc and Daguzan, 2000). In terms of sex, *S. officinalis* presented a similar composition of the diet for males and females (Guerra and Sánchez, 1985). Castro and Guerra (1990) observed that with the sexual maturation the feeding intensity of females increased. The variety of preys decreased with the increase of length (Castro and Guerra, 1990).

Predators of *Sepia officinalis* are marine mammals (Risso´s dolphin, Mediterranean monk seal and fur seals) and also, elasmobranchs (blue shark, smooth hound) (Pierce *et al.*, 2010).

I.5.7. Commercial importance, fisheries and aquaculture

The total global capture production in 2011 was the third ever, slightly below 1996 (93.8 million tons) and 2000 (93.5 million tons). Molluscs contributed with 767,021 tons in 2010 and 738,729 tons in 2011, and a slight reduction of 3.7% from 2011 to 2010 was observed (FAO, 2013). Global landings of cephalopods (cuttlefish, squid and octopus) have increased dramatically over the past 50 years and now constitute almost 5% of the total world’s fisheries production (Pierce *et al.*, 2010). The main reason for this increasing demand is that cephalopods are a good protein and lipid source (Kreuzer, 1984; Sinanoglou and Miniadis-Meimaroglou, 1998; Sinanoglou and Miniadis-Meimaroglou, 2000; Zlatanos *et al*., 2006), therefore a highly nutritious food that represents an alternative to over exploited fish resources (Sykes, 2009).

For cuttlefish, *Sepia officinalis* the FAO department of statistics presents the evolution of total landings of the specie, since 1950 until 2011 (Figure I.5). Since 1980 the landings of cuttlefish exceeded 100 000 t and were more than 116 000 t in 2006 (FAO statistic), comprising 49 000 t landed into Europe from the Northeast Atlantic and 67 000 t from Mediterranean. At the European scale, cuttlefish is currently the most important cephalopod fishery resource (Pierce *et al.*, 2010) and is presently the highest yielding cephalopod group harvested in the north-east Atlantic, with the English Channel supporting the main fishery for this species (Bloor *et al*., 2013).

Catches are made demersally by means of trawling and artisanal trapping. Trammel nets are widely used in most southern European countries for the exploitation of common cuttlefish. These traditional fisheries are seasonal and are related to the massive inshore spawning migration of *S. officinalis* (Pierce *et al.,* 2010). The operation of these nets to catch cuttlefish usually extends from late winter to early autumn, depending on the availability of the resource as well as the seasonal abundance of other commercial species (e.g. shrimp, sole) that are also targeted on inshore fishing grounds (Arnáiz *et al*., 2001; Lefkaditou *et al*., 2004). Traps are the dominant gear for the capture of cuttlefish in south Portugal, Algarve coast (Carneiro *et al.*, 2006).

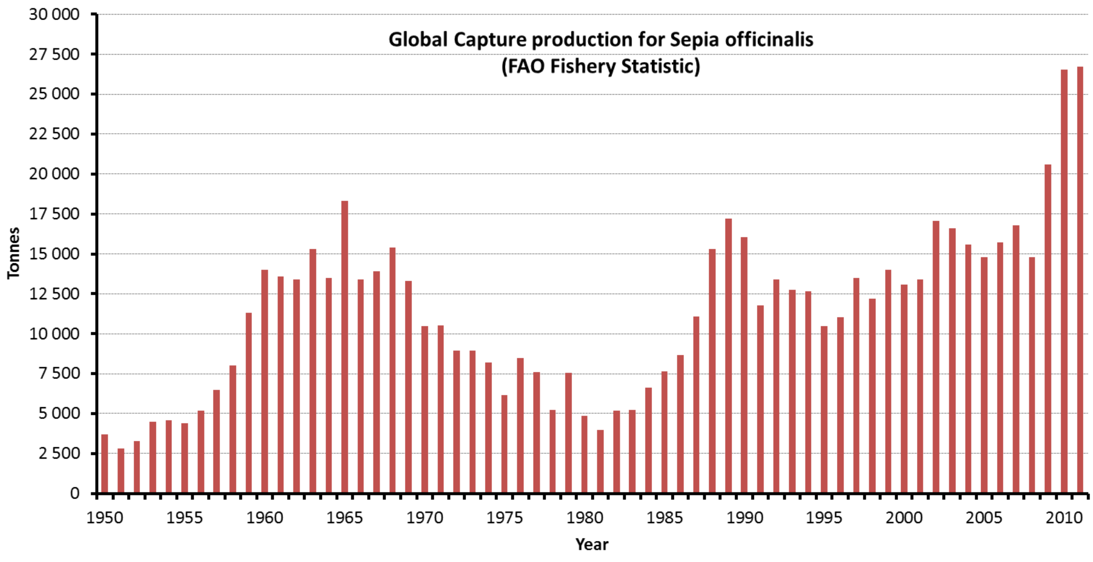

Figure I.5 - Total landings (tones) of cuttlefish (*Sepia officinalis*) in world since 1950 to 2011 (FAO statistics, 2013).

In Portugal, the Aveiro region has a key role in the Portuguese fisheries market, contributing with 10,695 t of fish, crustaceans and mollusks (DGPA, 2010). Cuttlefish is caught along the entire Portuguese coast being considered as an important fishery resource (Serrano, 1992). Local fishermen, in Ria the Aveiro, use trammel nets to capture cuttlefish (Jorge and Sobral, 2004). Despite being a seasonal fishery, cuttlefish landings in Aveiro Lagoon are very important to the local economy. Figure I.6 shows the total landings of cuttlefish between 2002 and 2012 and the annual turnover of those landings.

Along the years, landings of cuttlefish fluctuate from higher to lower catches, and this fact is certainly related with changes in environmental conditions and with the recruitment. Modelling and understanding cuttlefish exploitation is a challenge for a series of reasons: life-cycle and population dynamics are different from those of finfish (Caddy, 1983; Boyle and Boletzky, 1996). Recruitment, the renewal of harvestable stages in a population, is a key parameter of stock dynamics, and recruitment variability is often considered as the most difficult problem in stock assessment (Hilborn and Walters, 1992 in Challier *et al.* 2002). In Cephalopods, the short life span makes commercial catches more dependent on the number of recruits than in most teleost fish (Challier *et al.*, 2002). Early life stages are likely to incur higher mortalities than adult stages (Caddy, 1996), and are generally described as extremely responsive to environmental variables. Their population show wide fluctuations of abundance (Boyle and Boletzky, 1996), largely because they usually grow rapidly to maturity, they carry few food reserves (Rodhouse and Nigmatullin, 1996), have little overlap of generations and their migratory patterns make them particularly susceptible to chances in oceanographic conditions (Clarke, 1996). On the other hand, they show great resilience to fluctuations by their breeding seasons, varying the depth of their spawning grounds and maintaining complex recruitment patterns (Boyle and Boletzky, 1996).

Despite being seasonal, cuttlefish landings in the Ria de Aveiro are very important for the local economy (Figure I.6). In 2010, the annual turnover was more than 1.5 million Euros. Between 2002 and 2012 the average turnover was 846 thousand euros, and the average price for 1 Kg varied between 2.43 € and 3.72 € in 2004 and 2012, respectively.

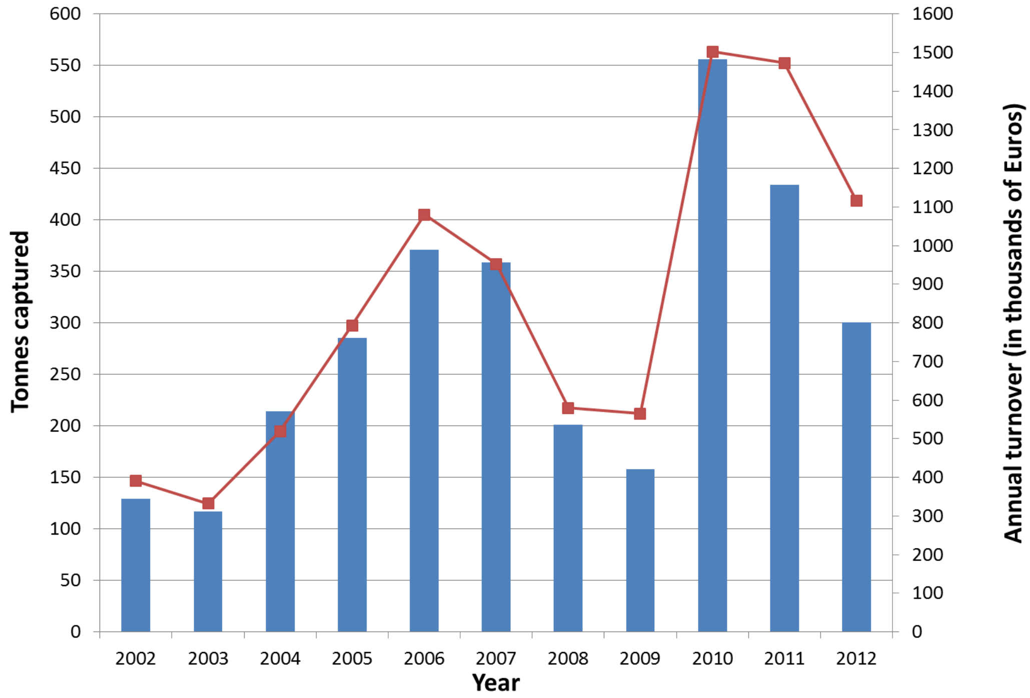

Figure I.6 - Total landings of cuttlefish in Aveiro Lagoon by tones captured (bars) and Annual turnover per year (line). (Docapesca - Statistical Department, 2013).

The high commercial value of cephalopods and their characteristics: short life cycles, fast growth rates and high food conversion, makes the aquaculture of this group of molluscs an area of increasing interest (Boletzky, 1983; Domingues *et al*., 2001a; Pierce *et al.*, 2010). The cuttlefish, *S. officinalis*, is an economically important species and therefore interesting to aquaculture (Pierce *et al*., 2010). *Sepia officinalis* is one of the most easily cultured cephalopods, and has been cultured in aquaria since the late 1960s (Richard, 1971; Pascual, 1978; Boletzky and Hanlon, 1983; Forsythe *et al*., 1994; Lee *et al*., 1998; Domingues *et al*., 2001a, 2001b, 2002, 2003, 2004a; Quintana *et al.*, 2005; Sykes *et al*., 2006; Baeza-Rojano *et al*., 2010). Aquaculture of cephalopods represents an alternative to over exploited fish resources, thus the increasing development in this area (Sykes, 2009).

I.6. Ria de Aveiro – a case study

According to INE (2011) the number of inhabitants surrounding the entire watershed is approximately one million people. Ria de Aveiro has an important environmental role in local and surrounding areas due to its variety of resources and uses such as fishing (sea-bass, eel, sole, flounder, lamprey, cuttlefish, crab, shrimp and cockle), aquaculture (mainly, sea-bass, eel, oyster and clam), recreational (windsurfing, boating, sailing and swimming) and traditional activities (salt production). Agriculture and cattle rearing, industries (chemical, metallurgic, ceramics, tannery and pulp miling), urban development and port activities increased significantly over years in the area around the lagoon (Abreu *et al.*, 1998; Pastorinho *et al*., 2012).

I.6.1. Morphologic and hydrological features

The Ria de Aveiro is a mesotidal lagoon located in the North-western of Portugal. It presents a very complex geometry and is characterized by large areas of intertidal flats and a web of narrow channels (Dias and Picado, 2011). The Ria de Aveiro lagoon constitutes a very important area, being the most extensive shallow lagoon system in Portugal and the most dynamic in terms of physical and biogeochemical processes (Picado *et al*., 2010). The lagoon covers an area of 83 km^2 at high tide (spring tide) and 66 km^2 at low tide, is 45 km long, 10 km wide and is connected to sea by a 350 m wide inlet, fixed by two jetties (Dias and Lopes, 2006). Connection to the sea was made artificially since 1808 (Pombo, 2005). Four main branches radiate from this sea entrance: Mira, S. Jacinto, Ílhavo and Espinheiro channels (Dias, 2009). This system is also characterized by a large number of other smaller channels between which lie significant intertidal areas, essentially mudflats, salt marshes and old salt pans (Picado *et al.*, 2010). The average depth of the lagoon relative to mean sea level is about 3 m (Picado *et al.*, 2010). The evolution process lasted for 800 years and occurred through the deposition of sand, with the formation of coastal sand dunes and a system of islands within the lagoon, this process was interrupted in the 18th century by human action (Silva, 2000).

The major factors that control the hydrologic dynamics of the Ria de Aveiro are:

a) Oceanic tide, which propagates from south to north along the west coast Portugal, entering in the lagoon by its mouth;
b) Winter that influences the lagoon for short periods and essentially in the wider part of the Ria de Aveiro;
c) Freshwater flows, transported by four major rivers that converge to the Ria: Vouga and Antuã from the East; Caster from the North and Boco from the Southeast (Dias *et al.*, 2001);

d) Wave regimes of north Atlantic also affect the morphodynamic of the Ria de Aveiro (Dias, 2009).

I.6.2. Contamination in Ria de Aveiro

The Ria de Aveiro is highly explored and impacted by anthropogenic activities, with significant and diverse sources of aquatic contaminants due to population settlement, industrial, agricultural and shipping activities (e.g. Pacheco *et al*., 2005; Vasconcelos *et al*., 2007; Pastorinho *et al*., 2012). The contamination pathways from anthropogenic sources, in the Ria de Aveiro, have been extensively documented (e.g. Monterroso *et al*., 2003a; Ramalhosa *et al*., 2006). Several works conducted in the Ria de Aveiro system, in northern Portugal, revealed high levels of several metals (Hg, Cu, Cd, Zn, Pb) (Borrego *et al.*, 1994; Martins, 2010; Monterroso *et al*., 2003a; Pereira, 2003; Nunes *et al*., 2008; Pastorinho *et al*., 2012).

Despite all the activities mentioned above the principal source of anthropogenic contamination in Ria de Aveiro is the chlor-alkali plant located in Estarreja, that started its activity in early decade of 1950 and presented different stages and phases of production increments (Pereira, 1997; OSPAR, 2005) and being responsible for the main Portuguese chlor and caustic production (OSPAR, 2005). As many studies show (e.g. Lacerda and Salomons, 1998 and Ullrich *et al*., 2007) mercury-cell chlor-alkali plants have been identified as main sources of mercury to the environment. Though the plant started to change the production process in 1994 and completely ceased the use of mercury in 2002 (Ospar Commission, 2006). Mercury emitted from the existing plant still remains significant in the surrounding environment. Additionally, it is known that through the years, large amounts of mercury have reached Ria de Aveiro, a nearby coastal lagoon, due to wastewater discharges (Reis, 2009) and a large number of abandoned mines draining to the Vouga River or its tributaries (Delgado *et al.*, 2000). Due to high levels of mercury found in Ria de Aveiro, this coastal lagoon and its environs have been extensively studied, with particular focus on sediments (Lucas *et al.*, 1986; Hall *et al.*, 1985; Pereira *et al.*, 1998; Pereira, 1997; Ramalhosa *et al.*, 2001, 2005, 2006; Ramalhosa *et al.*, 2006; Pereira *et al*., 2009; Pastorinho *et al.*, 2012), plankton (Monterroso *et al.*, 2003b), estuarine macroalgae (Coelho *et al.*, 2005) and aquatic organisms (Coelho *et al*., 2006; Válega *et al*., 2006; Nunes *et al.*, 2008; Cardoso *et al.*, 2013). An effort has been made to monitor species with economic importance, both invertebrate (Coelho *et al*., 2006; Coelho *et al.*, 2007; Pereira *et al*., 2006; Pereira *et al.*, 2009; Ahmad, 2012; Nilin *et al.*, 2012) and vertebrate species (Abreu *et al*., 2000).

I.7. Aims and Organization of the Thesis

The main objective of the present thesis was to characterize (biology, ecology, life cycle, diet and metal contamination) the cuttlefish, *Sepia officinalis*, in Ria de Aveiro, a resource of high importance and a current item of the humans' diet. In this thesis we pretended to answer to several questions such as: What is the distribution of cuttlefish in Ria de Aveiro? Which environmental factors affect the cuttlefish distribution in the Ria de Aveiro? Which is the spawning season of *Sepia officinalis* in the Ria de Aveiro? What are the feeding preferences of *Sepia officinalis* in the Ria de Aveiro? Is the diet of cuttlefish affected by season or sampling station? *Sepia officinalis* can accumulate metals in their tissues, and considering that Ria de Aveiro is a coastal ecosystem with a history of contamination, does the cuttlefish represents any risk for human health? What factors affected metal contamination in *Sepia officinalis* in Ria de Aveiro? To answer to these questions and others, several biological and ecological data was collected in Ria de Aveiro during this work. The choice of tissues analysed were selected according to previous studies (Miramand and Bentley, 1992) and the choice of sampling areas within the Ria de Aveiro were selected according to previous studies of ecology (Pombo and Rebelo, 2000) and ecotoxicology (Pereira *et al.*, 1998; Monterroso *et al*., 2003a).

The present thesis is organized in seven chapters. Each chapter can be summarized as follows:

- Chapter I: provides a theoretical introduction regarding aspects of contamination in aquatic ecosystems and in the studied area, and the characterization of *Sepia officinalis*;
- Chapter II: reviews the available bibliography concerning the biological, life cycle and ecological data about *Sepia officinalis* and makes a comprehensive study of this issues in Ria de Aveiro;
- Chapter III: describes the concentrations of essential metals (Fe, Zn, Cu) and non-essential (Cd and Pb) in mantle and digestive gland of *Sepia officinalis* in Ria de Aveiro and evaluates the seasonal and spatial trends of this accumulation.

It is also compared the obtained values with others European aquatic ecosystems and one in New Caledonia waters.

- Chapter IV: describes the concentrations of Hg in mantle and digestive gland of several selected sites in Ria de Aveiro and evaluates the eventual risk of consuming cuttlefish captured in a restrict area of Ria de Aveiro, the Laranjo bay.
- Chapter V: describes the levels of essential (Fe, Zn and Cu) and non-essential (Cd and Hg) in two tissues (mantle and digestive gland) during the life cycle of *Sepia officinalis*.
- Chapter VI: provides a general discussion of the results obtained in Chapters II-V. As each of these chapters include its own discussion material, in this chapter, only a concise and global discussion of results is presented in order to highlight synergies between the different chapters and to show the coherence of the work.

I.8. References

Abreu, S. N., Pereira, M. E., Duarte, A. C. (1998). The use of a mathematical model to evaluate accumulation in sediments and recovery time in a coastal lagoon (Ria de Aveiro, Portugal). *Water Science Technology*, 37(6-7): 33-38.

Abreu, S. N., Pereira, E., Vale, C., Duarte, A. C. (2000). Accumulation of Mercury in Sea Bass from a Contaminated Lagoon (Ria de Aveiro, Portugal). *Marine Pollution Bulletin*, 40(4): 293-297.

Alves, D. M., Cristo, M., Sendão, J., Borges, T. C. (2006) Diet of the cuttlefish *Sepia officinalis* (Cephalopoda: Sepidae) off the south coast of Portugal (eastern Algarve). *Journal of Marine Biology Association*, U.K., 86: 429-436.

Amiard, J. C., Geffard, A., Amiard-Triquet, C. (1998). La métallothionéine chez la moule Mytilus edulis comme biomarqueur de pollution métallique: variabilité entre sites, saisons et organes. *Journal de Recherche Oceanographique*, 23(1): 25–30.

Amiard-Triquet, C., Jeantet, A. Y., Berthet, B. (1993). Metal transfer in marine food chains: bioaccumulation and toxicity. *Acta Biologica Hungarica*, 44: 387-409.

Amiard-Triquet, C., Amiard, J.-C. (1998) Influence of ecological factors on accumulation of metal mixtures. *In*: Langston, W.J., Bebianno, M. (Eds.), Metal Metabolism in Aquatic Environments. Chapman and Hall, London, 351–386 pp.

Amiard, J.-C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P. S. (2006) Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology*, 76: 160–202.

Arnáiz, R., Rodrigues, E., Quintero, F., Bañón, R., Campelos, J.M., Gancedo, A., Tasende, M. *et al.* (2001). Lapesquería de *sépia* (*Sepia officinalis*) com trasmalhos en la enseñada de San Simón (1999-2000). *In*: Los Recursos Marinos de Galicia. Série Técnica Nº 2, Conselleria de Pesca e Assuntos Marítimos, Xunta de Galicia, Dirección Xeral de Recursos Mariños, Santiago de Compostela, Spain, pp. 54.

Baeyers, W., Leermakers, M., Pappina, T., Saprykin, A., Brion, N., Noyen, J. (2003). Bioconcentration and biomagnifications of mercury and methylmercury in the North Sea and Scheldt estuary fish. *Archives of the Environmental Contamination and Toxicology*, 45: 157-163.

Baeza-Rojano, E., García, S., Garrido, D., Guerra-García, J.M., Domingues, P. (2010) Use of Amphipods as alternative prey to culture cuttlefish (*Sepia officinalis*) hatchlings. *Aquaculture*, 300: 243–246

Barbosa, A., Mäthger, L. M., Buresch, K. C., Kelly, J. C., Chubb, Chiao, C.-C. *et al.* (2008). Cuttlefish camouflage: The effects of substrate contrast and size in evoking uniform, mottle or disruptive body patterns. *Vision Research*, 48: 1242-1253.

Bassaglia, Y., Buresi, A., Franko, D., Andouche, A., Baratte, S., Bonnaud, L. (2013) *Sepia officinalis*: A new biological model for eco-evo-devo studies. *Journal of Experimental Marine Biology and Ecology*, 447: 4–13.

Bebianno, M. J. (1990). Metallothionein induction and metal binding in *Littorina littorea* and *Mytilus edulis* on exposure to cadmium. PhD thesis. Zoology Department, University of Reading, UK, pp. 294.

Bebianno, M. J., Nott, J. A., Langston, W. J. (1993). Cadmium metabolism in the clam *Ruditapes decussatus*: The role of metallothionein. *Aquatic toxicology*, 27: 315-334.

Bebianno, M. J. and Langston, W. J. (1998) Cadmium and metallothionein turnover in different tissues of the gastropod *Littorina littorea*. Talanta, 46(2), 301-313.

Bebianno, M. J. and Serafim, M. A. (1998). Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *Science of the Total Environment*, 214(1-3): 123-131.

Bebianno, M.J. and Serafim, M.A. (2003). Variation of metal and metallothionein concentration in a natural population of *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology*, 214(1-3): 123-131.

Beeby, A. (2001). What do sentinels stand for? *Environmental Pollution*, 112, 285-298.

Bettencourt, V. (2000). Age and growth of cuttlefish, *Sepia officinalis* L. PhD. Thesis. University of Algarve, Portugal, pp196.

Blackmore, G. (1998). An overview of trace metal pollution in the coastal waters of Hong Kong. *Science of the Total Environment*, 214: 21-48.

Blanc, A., Pinczon du Sel, G., Daguzan, J. (1999). Relationships between length of prey/predator for the most important prey of the cuttlefish *Sepia officinalis* L. (Mollusca: Cephalopoda). Malacologia, 41: 139-145.

Blanc, A. and Daguzan, J. (2000). Size selectivityin the diet of the young cuttlefish *Sepia officinalis* (Mollusca:Sepiidae). *Journal of Marine Biology Association*, UK, 80, 1137-1138.

Bloor, I. S. M., Attrill, M. J., Jackson, E. L. (2013). Chapter One – A Review of the Factors Influencing Spawning, Early Life Stage Survival and Recruitment Variability in the Common Cuttlefish (*Sepia officinalis*). Advances in Marine Biology 65: 1–65.

Boal, J. G., Hylton, R. A., González, S. A., Hanlon, R. T. (1999). Effects of crowding on the social behavior of cuttlefish (*Sepia officinalis*). *Contemporary* Topics in *Laboratory Animal Science*, 38(1): 49-55.

Boletzky, S. V. (1979). Growth and life-span of *Sepia officinalis* under artificial conditions. *Rapport Comunitaire International Mer Mèditeranee*, 10, 10 p.

Boletzky, S. V. (1983). *Sepia officinalis*. *In*: Boyle, P.R. (Ed.), *Cephalopod Life Cycles*, vol.1. Academic Press, London, pp.31-52.

Boletzky, S. V. and Hanlon, R. (1983). A review of the laboratory maintenance, rearing and culture of cephalopod mollusks. *Memoirs of the National Museum of the National Museum Victoria*, 44: 147-187.

Boletzky, S. V. (1988). A new record of long-continued spawning in *Sepia officinalis* (Mollusca, Cephalopoda). *Rapport Commission Internationale pour la Mer Mediterranee*, 31, 257 p.

Borrego, C., Cruz, M. M., Gonçalves, P. (1994). Estudo de avaliação da vulnerabilidade da capacidade de recepção das águas costeiras em Portugal. Relatório R 3.2 Meios receptores e suas características: (Be 1) – Ria de Aveiro, Pateira de Fermentelos e Barrinha de Mira. *Projecto nº 13/94. IDAD*, Aveiro.

Boucaud-Camou, E., Yim, M., Tresgot, E. (1985). Feeding and digestion of young *Sepia officinalis* L. (Mollusca: Cephalopoda) during post-hatching development. *Vie Milieu*, 35(3/4): 263-266.

Boucher-Rodoni, R., Boucaud-Camou, E., Mangold, K. (1987). Feeding and digestion. *In*: Cephalopod life cycles. Vol.II. Comparative reviews (ed. P. Boyle), pp. 85-108. London: Academic Press.

Boucaud-Camou, E. (1990). La seiche, un animal d’avenir. La Pêche Maritime. 1342: 321-329.

Boucaud-Camou, E. and Boismery, J. (1991). The migration of the cuttlefish (*Sepia officinalis L.*) *In*: La Sheiche: the Cuttlefish, 1st International Symposium on the Cuttlefish *Sepia*, 1-3june 1989, Caen, pp. 179-189. Ed. by Boucaud-Camou E., Editor. *Centre de Publications de l’Université de Caen*, Caen, FranceThe Cuttlefish, Université de Caen, pp.358.

Boyle, P.R. and Boletzky, S.V. (1996) Cephalopod populations: definition and dynamics. *Philosophical transactions of the Royal Society of London*, 351: 985-1002.

Brito, H.V. (1998). Estudo de alguns aspectos da biologia reprodutiva da espécie *Sepia officinalis* (Linnaeus, 1758) na Ria de Formosa. MSc thesis, University of Algarve, pp. 82.

Brown, M.T. and Depledge, M.H. (1998) Determinations of trace metal concentrationin marina organisms. *In*: Langston, W.J., Bebiano, M.J. (Eds.), Metal Metabolism in aquatic Environments. Chapman and Hall, London, 219-283.

Bryan, G.W. (1979). Bioaccumulation of marine pollutants. *Philosophical Transactions of the Royal Society of London*, series B: Biological Sciences, 286: 483-505 *In*: Blackmore, G. (1998). *An overview of trace metal pollution in the coastal waters of Hong Kong. Science of the Total Environment*, 214: 21-48.

Bryan, G. W., Langston, W. J., Hummerstone, L. G. (1980). The use of biological indicators of heavy metal contamination in estuaries: with special reference to an assessment of the biological availability of metals in estuarine sediments from south-west Britain. *Occasional Publication Marine Biological Association of the United Kingdom*, 1: 1-73.

Bustamante, P. (1998). Etude des processos de bioaccumulation et de détoxication d`élément traces (métaux lourds et terres rares) chez les mollusques céphalopodes et bivalves pectinidés. Implication de leur biodisponibilité pour le transfert vers les prédateurs. Tese de Doutoramento, Universidade de La Rochelle, 290 p.

Bustamante, P, González, A. F., Rocha, F., Miramand, P., Guerra, A. (2008). Metal and metalloid concentrations in the giant squid *Architeuthis dux* from Iberian Waters. *Marine Environmental Research*, 66: 278-287.

Butler, P. A., Andren, L., Bonde, J., Jernelov, A., Reisch, D. J. (1971). Monitoring organisms. *In*: FAO Technical Conference on Marine Pollution and its Effects on Living Resources and Fishing, Rome, 1970. Suppl. 1: Methods of Detection, Measurement and Monitoring of Pollutants in the Marine Environment. *FAO Fisheries Reports*, 99(S1): 101-112.

Caddy, J. F. (1983). The cephalopods: factors relevant to their population dynamics and to the assessment and management of stocks. *In*: Caddy, J.F. (Ed.), Advances in Assessment of World Cephalopods Resources. *FAO Fisheries Technical Paper*, Rome, (231): 416–452.

Caddy, J. F. (1996). Modelling natural mortality with age in short-lived invertebrate populations: definition of a strategy of gnomonic time division. *Aquatic Living Resources*. 9: 197–207.

Cairns, J. and Van der Schalie, W. H. (1980). Biological Monitoring. Part I – Early warning systems. *Water Research*, 14(9): 1179-1196.

Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Science of the Total Environment*, 247, 295-311.

Cardoso, P. G., Sousa, E. P., Matos, P., Henriques, B., Pereira, E., Duarte, A.C., Pardal, M.A. (2013). Impact of mercury contamination on the population dynamics of *Peringia ulvae* (Gastropoda): Implications on metal transfer through the trophic web. Estuarine, *Coastal and Shelf Science*, 129: 189–197.

Carneiro, M.C., Martins, R., Rebordão, F.R. (2006). Contribuição para o conhecimento das artes de pesca utilizadas no Algarve. *Publicações Avulsas do IPIMAR*, 13: 60.

Castro, B. G. and Guerra, A. (1989). Feeding pattern of *Sepia officinalis* (Cephalopoda: Sepiodidea) in the Ria de Vigo (NW Spain), *Journal of marine biological association*, UK. 69: 545-553.

Castro, B.G. and Guerra, A. (1990). The diet of *Sepia officinalis* and *Sepia elegans* (Cephalopoda, Sepioidea) from the Ría de Vigo. *Scientia Marina*, 54(4): 375-388.

Challier, L., Royer, J, Robin, J-P. (2002). Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. *Aquatic Living Resources*. 15, 303-311.

Chapman, P.M., Wang, F., Janssen, C.R., Goulet, R.R., Kamunde, C.N. (2003). Conducting ecological risk assessment of inorganic metals and metalloids: current status. *Human and Ecological Risk Assessment*, 9: 641-697.

Chiao, Chuan-Chin, Chubb, C., Buresh, K., Siemann, L., Hanlon, R.T. (2009). The scaling effects of substrate texture on camouflage patterning in cuttlefish. *Vision Research*, 49 (13): 1647-1656.

Clarke M.R. (1996). The role of cephalopods in the world's oceans: general conclusions and the future. *Philosophical Transactions of the Royal Society of London*, 351: 1105-1112.

Coelho J.P., Pereira E., Duarte A., Pardal M. (2005). Macroalgae response to a marcury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal). *Estuary Coastal Shelf Science Journal*, 65: 492-500.

Coelho J.P., Rosa M., Pereira E., Duarte A., Pardal M.A. (2006). Pattern and annual rates of *Scrobicularia plana* mercury bioaccumulation in a human induced mercury gradient (Ria de Aveiro, Portugal). *Estuarine, Coastal and Shelf Science,* 69: 629-635.

Coelho J.P., Policarpo E., Pardal M.A., Millward G.E., Pereira M.E., Duarte A.C. (2007). Mercury contamination in invertebrate biota in a temperate coastal lagoon (Ria de Aveiro, Portugal). *Marine Pollution Bulletin*, 54: 475-80.

Connell, D.W. and Miller, G.J. (1984). Chemistry and Ecotoxicology of Pollution, pp. 464. ISBN: 978-0-471-86249-9

Correia, M., Domingues, P.M., Sykes, A., Andrade, J.P. (2005). Effects of culture density on growth and broodstock management of the cuttlefish, *Sepia officinalis* (Linnaeus, 1758), *Aquaculture*, 245(1-4): 163-173.

Delgado, H., Rocha, F., Gomes, C. (2000). Modelos de dispersão de metais pesados nos sedimentos recentes da laguna de Aveiro e sua relação com a composição e textura dos sedimentos. *In*: Duarte, A., Vale, C., Prego, R. (eds) Estudos de Biogeoquímica na Zona Costeira Ibérica (actas IX Seminário Ibérico de química Marinha), Universidade de Aveiro, Portugal, 325-336.

Depledge, M.H. and Rainbow, P.S. (1990). Models of regulation and accumulation of trace elements in marine invertebrates: a mini-review. *Comparative Biochemistry and Physiology*, C 97: 1-7.

DGPA. Recursos da Pesca. (2010). *In*: Aquicultura D-GdPe, editor. 23 A-B. Lisboa: Direção-Geral das Pescas e Aquicultura; pp. 182.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A. (1995) Biochemical mechanisms: metabolism, adaptation, and toxicity. *In*: Rand., G. M. (Ed.), *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*, Second Edition. Taylor and Francis, Washington, 523-561.

Dias, J., Lopes, J., Dekeyser, I. (2001). Lagrangian transport of particles in Ria de Aveiro lagoon, Portugal. *Physics Chemistry Earth Part B: Hydrology Oceans Atmosphere*, 26: 721-727.

Dias, J.M. (2001). Contribution to the Study of Ria de Aveiro Hydrodynamics. PhD thesis. Physical Department, University of Aveiro, Portugal, pp. 288. http://nmec.web.ua.pt/ficheiros/PDFs/jdias.pdf

Dias, J.M. and Lopes, J.F. (2006). Implementation and Assessment of Hydrodynamic, Salt and Heat Transport Models: The case of the Ria de Aveiro Laggon (Portugal). *Environmental Modelling & Software*. 21: 1-15.

Dias, J.M. (2009). Hidromorfologia da Ria de Aveiro:alterações de origem antropogénica e natural. Debater a Europa - Periódico do CIEDA e do CIEJD, em parceria com GPE, RCE e o CEIS20. Available at: http://www.europe-direct-aveiro.aeva.eu/debatereuropa/

Dias, J.M., Picado, A. (2011). Impact of morphologic anthropogenic and natural changes in estuarine tidal dynamics. *Journal of Coastal Research*, 64: 1490-1494.

DocaPesca of Aveiro. (2013). Total landings of cuttlefish in Aveiro Lagoon by tones captured and annual turnover per year, since 2002 to 2012. Statistical Department.

Domingues, P.M., Kingston, T., Sykes, A., Andrade, J.P. (2001a). Growth of young cuttlefish, *Sepia officinalis* (Linnaeus, 1758) at the upper end of the biological distribution temperature range. *Aquaculture Research*, 32: 923-930.

Domingues, P.M.T., Sykes, A., Andrade, J.P. (2001b). The use of Artemia or mysids as food for hatchlings of the cuttlefish *Sepia officinalis* Linnaeus, 1758; effects on growth and survival throughout the life cycle. *Aquaculture International*. 9: 319– 331.

Domingues, P.M.T., Sykes, A., Andrade, J.P. (2002). The effects of temperature in the life cycle of two consecutive generations of the cuttlefish *Sepia officinalis* (Linnaeus, 1758), cultured in the Algarve (South Portugal). Aquac. Int. 10, 207–220.

Domingues, P.M., Poidier, R., Dickel, L., Almansa, E., Sykes, A., Andrade, J.P. (2003). Effects of culture densities and live prey on growth and survival of juvenile cuttlefish, *Sepia officinalis*. *Aquaculture International*, 11: 225-242.

Domingues, P., Sykes, A., Sommerfield, A., Almansa, E., Lorenzo, A., Andrade, J.P. (2004a). Growth and survival of cuttlefish (*Sepia officinalis*) of different ages fed crustaceans and fish. Effects of frozen and live prey. *Aquaculture*, 229: 239-254.

Domingues, P.M., Cortés, G.G., Vásquez, C.R. (2004b). Alimentación y nutrición de Moluscos Céfalopodos: Avances recientes y Perspectivas Futuras. *In*: Cruz Suárez, L.E., Ricque Marie, D., Nieto Lòpez, M.G., Villareal, D., Scholz, U. y González, M. Avances en Nutrición Acuícola VII. Memorias del VII Simposium Internacional de de Nutrición acuícola. Hermosilo, Sonora, México, 16-19.

FAO, (2013). FAO statistical yearbook 2013: World Food and Agriculture. Food and Agriculture organization of the United Nations, Rome.

Fernandes, C., Fontaínhas-Fernandes, A., Peixoto, F., Salgado, M.A. (2007). Bioaccumulation of heavy metals in *Liza saliens* from the Esmoriz-Paramos coastal lagoon, Portugal. *Ecotoxicology Environment Safety*, 66: 426-431.

Fichet, D., Boucher G., Radenac, G., Miramand, P. (1999). Concentration and mobilization of Cd , Cu, Pb and Zn by meiofauna populations living in harbour sediment: their role in the heavy metal flux from sediment to food web. *Science of the Total Environment*, 243/244: 263-272.

Finger, J.M. and Smith, J.D. (1987). Molecular association of Cu, Zn, Cd, and 210Po in the digestive gland of the squid *Nototodarus gouldi*. *Marine Biology*, 65: 87-91.

Forsythe, J., DeRusha, R., Hanlon, R. (1994). Growth, reproduction and life span of *Sepia officinalis* (Cephalopoda: Mollusca) cultured through seven consecutive generations. *Journal of. Zoology*. London. 233:175–192.

Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B., Depledge, M.H. (2004). Ecossystem management bioindicators: the ECOMAN project - a multibiomarker approach to ecosystem management. *Marine Environmental Research*, 58: 233-237.

Gauvrit, E., Le Goff, R., Daguzan, J. (1997). Reproductive cycle of the cuttlefish, *Sepia officinalis* (L.) in the Northern part of the Bay of Biscay. *Journal of Molluscan Studies*, 63: 19-28.

Goldberg, E. D., Koide, M., Hodge, V., Flegal, A. R., & Martin, J. (1983). US Mussel Watch: 1976-1978

Guerra, A. and Sánchez, P. (1985). Comparación de las pesqueiras de cefalópodos de los áreas de afloramiento de la costa occidental africana. *Simpo Intern Aflor Inst Inves Pesq Barcelona* II: 749-760.

Guerra, A. (1987). La *Sepia* un invertebrado muy desarrollado. *Revista oficial de la Junta del Puerto y Ria de Vigo*, Nº X, 30-33.

Guerra, A. and Castro, G.B. (1988). On the life cycle of *Sepia officinalis* (Cephalopoda, Sepioidea) in the Ria de Vigo (NW Spain). *Cahiers de Biologie Marine*, 29: 395-405.

Guerra, A. (1992). Mollusca, Cephalopoda. Fauna Ibérica. M.A. Ramos, Ed.). Vol.1. Museu Nacional de Ciencias Naturales. Consejo Superior de Investigaciones Cientificas, Madrid, pp. 327.

Guerra, A., Perez-Losada, M., Rocha, F. (2001) Species differentiation of *Sepia officinalis* and *Sepia hierredda* (Cephalopoda: Sepiidae) based on morphological and allozyme analyses. *Journal of the Marine Biological Association of the UK*, 81: 271-281.

Guerra, A. (2006). Ecology of *Sepia officinalis*. Vie et Milieu – *Life & Environment*. 56(2): 97-107.

Hall, A., Lucas, M.F., Caldeira, M.T., Duarte, A.C. (1985). Distribuição de Mercúrio nos Sedimentos da Ria de Aveiro (Distribution of Mercury in the sediments of Ria de Aveiro, Portugal). Actas das jornadas da Ria de Aveiro. Poluição da Ria de Aveiro, I. Câmara de Aveiro, pp.103-25.

Hamelink, J.L., Landrum, P.F., Bergman, H.L., Benson, W.H. (1994). Bioavailability: Physical, Chemical, and Biological Interactions. Lewis, Boca Raton, FL.

Hanlon, R. T., and Messenger, J. B. (1988). Adaptive coloration in young cuttlefish (*Sepia officinalis* L.): The morphology and development of body patterns and their relation to behaviour. *Philosophical Transactions of the Royal Society of London B*, 320: 437–487.

Hilborn, R., Walters, R.F. (1992). Quantitative Fish Stock Assessment: Choice, Dynamics and Uncertainty. Chapman & Hall, New York.

Hudson, R. (1998). Which aqueous species control the rates of trace metal uptake by aquatic biota? Observations and predictions of non-equilibrium effects. *Science of the Total Environment*, 219: 95-115.

Hylland, K., Ruus, A., Børseth, J.F., Bechmann, R., Baršienė, J., Grung, M., Tollefsen, K., Myhre, L.P. (2006). Biomarkers in monitoring - a review. NIVA-report 5205. 106 p.c

INE (2011). Instituto Nacional de Estatística. Censos 2011: resultados provisórios.

Isani, G., Cortesi, P., Cattani, O., Viviani, R., Carpené, E. (2000). Biochemical responses in specimens of *Sparus aurata* fed experimental diets containing variable protein sources. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 126: 23

Jorge, I. and Sobral, M.P. (2004). Alguns aspectos da biologia e da ecologia do choco, *Sepia officinalis* (Linnaeus, 1758), da Ria de Aveiro e do Litoral adjacente. *Relatório Técnico-Científico do IPIMAR, Série digital* (http://ipimar-iniap.ipimar.pt), nº 15, 29 p.

Kowalshi, P.A.O.P. (2009). Combinated use of chemical data and biomarkers in aquatic key species – An ecotoxicological study in Óbidos lagoon. PhD thesis. Biology Department, University of Aveiro, Portugal, pp. 294.

Kreuzer, R. (1984). Cephalopods: handling, processing and products. *FAO Fisheries Technical Paper*, pp. 254.

Kromov, D.N., Lu C.C., Guerra A., Dong Zh, Boletzky S.V. (1998). A synopsis of Sepiidae outside Australian waters, Smith. *Contributions to Zoology*, 586: 77-157.

Lacerda, L. and Salomons, W. (1998). Mercury from gold and silver mining: a chemical time bomb? Berlin: Springer-Verlag; p. 146.

Lall, S.P. (1995) Macro and trace elements in fish and shellfish. In A.Ruiter (Ed.), *Fish and fishery products. Composition, nutritive properties and stability* (pp. 187-213). Wallingford: Cab International.

Langston, W.J., Bebianno, M.J., Burt, G.R. (1998). Metal handling strategies in molluscs. In Langston, W.J., Bebianno, M.J. (Eds.), *Metal Metal metabolism in Aquatic Environments.* Chapman and Hall, London, pp. 219-283.

Langston, W.J. and Spence, S.K. (1995). Biological factors involved in metal concentrations observed in aquatic organisms. *In*: Tesseir, A., Turner, D.R. (Eds.), *Metal speciation and bioavailability in aquatic systems*. New York, John Wiley, pp. 407-478.

Lee, C., L., Fang, M.D., Hsieh, M.T. (1998). Characterization and distribution of metals in surficial sediments in Southwestern Taiwan. *Marine Pollution. Bulletin*, 36 (6): 464–471.

Lefkaditou, E., Haralabous, J., Sarikas, D., Karamelidou, S., Kavadas, S. (2004). The Cephalopods in the small-scale fishery in the eastern Thracian Sea (NE Mediterranean). ICES Document CM 2004/CC:19, 12pp.

Li, Y H., Burkhardt, M., Terakoa, H. (1984) Desorption and coagulation of trace elements during estuarine mixing. *Geochimica and Cosmochimica*, Acta 48, 201 l-2025.

Lopes, C.L., Silva, P.A., Dias, J.M., Alfredo, R., Picado, A., Plecha, S., Fortunato, A.B. (2011). Local sea level change scenarios for the end of the 21 st century and potencial physical impacts in the lower Ria de Aveiro (Portugal). *Continental Shelf Research*, 31: 1515-1526.

Lucas, M.F., Caldeira, M.T., Hall, A., Duarte, A.C., Lima, C. (1986). Distribution of mercury in the sediments and fishes of the lagoon of Aveiro, Portugal. *Water Science Technology*, 18: 141-148.

Lueng, K.M., Furness, R.W. (2001). Metallothionein induction and condition index of dogwhelks *Nucella lapillus* (L.) exposed to cadmium and hydrogen peroxide. *Chemosphere*, 44: 321-325.

Mangold-Wirz K. (1963). Biologie des Céphalopodes bentiques et nectoniques de la Mer Catalane. *Vie Milieu*, suppl. 13: 1-285.

Mangold K. (1966). Sepia officinalis de la mer Catalane.Vie et Milieu, 17:961-1012.

Martins, M.C. (1990). Revisão da espécie *Sepia officinalis, 1758* e um estudo preliminar da sua biologia e pesca na região algarvia. Relatório de Estágio da Licenciatura de Biologia Marinha e Pescas, Universidade de Faro, 70 p.

Martins, V., Ferreira da silva, E., Sequeira, C., Rocha, F., Duarte, A.C., (2010). Evaluation of the ecological effects of heavy metals on the assemblages of benthic foraminifera of the canals of Aveiro (Portugal). *Estuarine, Coastal and Shelf Science*, 87: 293–304.

McCarthy, JF, Shugart, LR. (1990). Biological markers of environmental contamination. In: McCarthy JF, Shugart LR, editors. *Biomarkers of environmental contamination*. Boca Raton, FL: Lewis Publishers, 3-14.

McLusky, D.S., Bryant, V., Campbell, R. (1986). The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. Oceanography and Marine Biology: An Annual Review, 24: 481-520.

Mcluscky, D.S. and Elliot, M. (2004). The Estuarine Ecosystem: ecology, threats and management. (3rd Edition). Oxford University Press, Oxford.

Miao, S., Delaune, R.D., Jugsujinda, A. (2006). Influence of sediment redox conditions on release/solubility of metals and nutrients in Louisiana Mississipi River deltaic plain freshwater lake. *Science of the Total Environment*, 371: 334-343.

Miramand, P. and Bentley, D. (1992). Concentration and distribution of heavy metals in tissues of two cephalopods, *Eledone cirrhosa* and *Sepia officinalis*, from the French coast of the English Channel. *Marine Biology*, 114, 407-414.

Monteiro, L.R., Porteiro, F.M., Gonçalves, J.M. (1992). Inter- and intra-specific variation of mercury levels in muscle of cephalopods from the Azores. *Arquivos de Ciencias do Mar*, 0 (10): 13-22.

Monterroso, P., Pato, P., Pereira, E., Vale, C., Duarte, A.C. (2003a). Distribution and accumulation of metals (Cu, Cd, Zn and Pb) in sediments of a lagoon on the northwestern coast of Portugal. *Marine Pollution Bulletin*, 46: 1200-1211.

Monterroso, P., Abreu, *S*.N., Pereira, E., Vale, C., Duarte, A.C. (2003b). Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro). *Acta Oecologica*, 24:S351-S357.

Nash, R.D.M. and Thorpe, J.P. (2003). MARINE FOODS | Edible Animals Found in the Sea. Encyclopedia of Food Sciences and Nutrition (Second Edition). 3728-3733.

Nieboer, E. and Richardson, D.H.S. (1980). The replacement of the nondescript term ‘heavy metals’ by a biologically and chemically significant classification of metal ions. *Environmental Pollution B*, 1: 3-26.

Nilin, J., Pestana, J.L.T, Ferreira, N.G., Loureiro, S., Costa-Lotufo, L. V., Soares, A. M. V. M. (2012). Physiological responses of the European cockle *Cerastoderma edule* (Bivalvia: Cardidae) as indicators of coastal lagoon pollution. *Science of the Total Environment*, 435–436: 44–52.

Nixon, M. and Mangold, K. (1998). The early life of *Sepia officinalis*, and the contrast with that of *Octopus vulgaris* (cephalopoda). *Journal of Zoology*, London 254: 401-425.

Nobre, A. (1931). Moluscos Marinhos de Portugal. 1º Volume, Imprensa Portuguesa, Porto, pp. 466.

Nordberg, M., Douglas, M.T., Andersen, O., Duffus, J.H. (2009). Glossary of terms used in ecotoxicology (IUPAC Recommendations) *Pure Applied Chemistry*, 81(5): 829-970.

Norman, M. (2000). Cephalopods – A world Guide, Conchbooks, Germany.

Nunes, M., Coelho, J.P., Cardoso, P.G., Pereira, M.E., Duarte, A.C., Pardala, M.A. (2008). The macrobenthic community along a mercury contamination in a temperate estuarine system (Ria de Aveiro, Portugal). *Science of the Total Environment*, 405: 186-194.

Okutani, T. (2005). Cuttlefish and Squids of the world 2005 in http://www.zen-ika.com.

OSPAR Commission (2000). Quality status Report 2000: Region IV – Bay of Biscay and Iberian Coast, OSPAR Commission, London. 134 + XIIIpp.

OSPAR Commission (2005). Mercury losses from the chlor-alkali industry (1992-2003). Hazardous Substances Series.

OSPAR Commission (2013).Chapter 10: Metallothionein (MT) in blue mussels (*Mytilus edulis, Mytilus galloprovincialis*). Monitoring and Assessment Series, 91-96.

Pacheco, M., Santos, M.A., Teles, M., Oliveira, M., Rebelo, J.E., Pombo, L. (2005). Biotransformation and genotoxic biomarkers in mullet species (*Liza* sp.) from a contaminated coastal lagoon (Ria de Aveiro, Portugal). *Environmental. Monitoring and Assessment*, 107: 133–153.

Palmegiano, G.B. and D'Apote, M.P. (1983). Combinated effects of temperatura and salinity on cuttlefish (*Sepia officinalis* L.) hatching. *Aquaculture*, 35: 259-264.

Pan, L. and Zhang, H. (2006). Metallothionein, antioxidant enzymes and DNA strand breaks as biomarkers of Cd exposure in a marine crab, *Charybdis japonica*. *Comparative Biochemistry and Physiology*, C.144: 67-75.

Pascual, E. (1978). Crescimiento y alimentación de tres generaciones de *Sepia officinalis* en cultivo. Investigacion Pesquera. 42: 421–442.

Pastorinho, M.R., Telfer, T.C., Nogueira, A.J.A., Soares, A.M.V.M., Ranville, J.F. (2012). An evaluation of trace metal distribution, enrichment factos and risk in sediments of a coastal lagoon (Ria de Aveiro, Portugal). *Environmental Earth Science,* DOI: 10.1007/s12665-012-1643-xOnline First™.

Peakal, D.B. (1994). The role of biomarkers in the environmental assessment (1) Introduction. *Ecotoxicology*, 3, 157-160.

Pereira, M.E. (1997). Distribution, reactivity and transport of mercury in the Aveiro lagoon, PhD thesis. Departamento de Química, Universidade de Aveiro.

Pereira, M.E., Duarte, A.C., Millward, G., Abreu, S.N., Vale, C. (1998). An estimation of industrial mercury stored in sediments of a confined area of the lagoon of Aveiro (Portugal). Water Science Technology 37(6-7), 125-130.

Pereira, P.A.O. (2003). Repartição de metais e organoclorados em *Sepia officinalis* de dois sistemas lagunares: Ria de Aveiro e Ria Formosa. MSc thesis. Departamento de Biologia, Universidade de Aveiro, pp. 96 + XXII

Pereira, E., Abreu, S.N., Coelho, J.P., Lopes, C.B., Pardal, M.A., Vale, C., Duarte, A.C. (2006). Seasonal fluctuations of tissue mercury contents in the european shore crab *Carcinus maenas* from low and high contamination areas (Ria de Aveiro, Portugal). *Marine Pollution Bulletin*; 52: 1450-1457.

Pereira, P., Raimundo, J., Vale, C., Kadar, E. (2009). Metal concentrations in digestive gland and mantle of *Sepia officinalis* from two coastal lagoons of Portugal. *Science of the total Environment*, 407: 1080-1088.

Phillips, D. J. H. and Rainbow, P. S. (1988). Barnacles and mussels as biomonitors of trace elements: a comparative study. *Marine Ecology Progress Series*, 49: 83-93.

Phillips, D. J. H. (1990) Use of macroalgae and invertebrates as monitors of metal levels in estuaries and coastal waters. *In*: *Heavy Metals* in the Marine Environment (R. W. Furness & P. S. Rainbow, eds), pp. 81-99. CRC Press, Boca Raton, FL.

Phillips, D. J. H. and Rainbow, P. S. (1993). Biomonitoring of Trace Aquatic Contaminants. *Applied Science Publishers*, Barking.

Picado, A., Dias, J.M., Fortunato, A. (2010). Tidal changes in estuarine systems induced by local geomorphologic modifications. *Continental Shelf Research*, 30(17): 1854-1864.

Pierce, G. J., Allcock, L., Bruno, I., Bustamante, P., González, Á., Guerra, Á., Jereb, P., *et al*. (2010). Cephalopod biology and fisheries in Europe. *ICES Cooperative Research Report* Nº303. pp. 175.

Pimenta, J.R. (2010). Accumulation, responses and genotoxicity of trace elements in *Octopus vulgaris*. PhD thesis. Biochemistry Department, University of Lisbon, Portugal, p. 176.

Pinczon du Sel, G. and Daguzan, P.S. (1992). Contribution a la connaissance du regime alimentaire de la seiche (*Sepia officinalis* L.) (Mollusque, Cephalopode) dans le Nord du Golfe de Gascogne et dans le golfe du Morbihan: Resultats preliminaires. *Les Mollusques Marins Biologique et Aquaculture*, 14: 155-172.

Pinczon du Sel, G., Blanc, A., Daguzan, J. (2000). The diet of the cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda) during its life cycle in the Northern Bay of Biscay (France). *Aquatic Sciences* 61: 167–178.

Point, D., Monperrus, M., Tessier, E., Amouroux, D., Chauvaud, L., Thouzeau, G., Jean, F., Amice, E., Grall, J., Leynaert, A., Clavier, J., Donard, O.F.X. (2007). Biological control of trace metal and organometal benthic fluxes in a eutrophic lagoon (Thau Lagoon, Mediterranean Sea, France). *Estuarine Coastal Shelf Science*, 72: 457-471.

Pombo, L. and Rebelo, J.E. (2000). Ria de Aveiro ictiofauna: diversity in space and time. *Revista da Biologia*, Lisboa, 18: 69-81.

Pombo, L. (2005). Diversity, population dynamics and fish production capacity in a coastal lagoon – the Ria de Aveiro. Ph.D. Thesis, Biology Department, University of Aveiro, Portugal, pp. 272.

Quintana, D., Márquez, L., Almansa, E., Rodríguez, E., Lorenzo, A. (2005). Efecto de la dieta suministrada a reproductores de pulpo común (Octopus vulgaris) sobre la calidad de puesta y la composición bioquímica de huevos y paralarvas. Comunication Oral, X Congresso Nacional de Acuicultura, Gandía (Valencia).

Rabouille, C., Amouroux, D., Anschutz, P., Jouanneau, J.-M., Gilbert, F., Cossa, D., Prevot, F. (2007). Biogeochemical and contaminant cycling in sediments from a human-impacted coastal lagoon – introduction and summary. *Estuarine, Coastal and Shelf Science*, 72(3): 387-392.

Rainbow, P. S. (1990). Heavy metal levels in marine invertebrates. *In*: *Heavy Metals in the Marine Environment.* (R. W. Furness & P. S. Rainbow, eds), pp. 67-79. CRC Press, Boca Raton, FL.

Rainbow, P. S. (1993). Biomonitoring of marine heavy metal pollution and its application in Hong Kong waters. In The Marine Biology of the South China Sea. Proc. Ist Int. Conf. on the Marine Biology of Hong Kong and the South China Sea, Hong Kong 1990. (B. Morton, ed.), pp. 235-250. Hong Kong University Press, Hong Kong.

Rainbow, P. S. and Phillips, D. J. H. (1993). Cosmopolitan biomonitors of trace metals. *Marine Pollution Bulletin*, 26: 593-601.

Rainbow, P.S. (1995). Biomonitoring of Heavy Metal Availability in the Marine Environment. *Marine Pollution Bulletin,* 31: 183-192.

Rainbow, P.S. (1997a). Trace metal accumulation in marine invertebrates: marine biology or marine chemistry. *Journal of the Marine Biological Association of the United Kingdon*, 77: 195-210.

Rainbow, P.S. (1997b). Ecophysiology of trace metal uptake in crustaceans. *Estuarine, Coastal and Shelf Science*, 44: 169-175.

Rainbow, P.S. (2002). Trace metals concentrations in aquatic invertebrates: why and so what? Environment Pollution: Models, metabolic availability and toxicity. *Environmental Pollution*, 120: 497-507.

Rainbow, P.S. (2007). Trace metal bioaccumulation: Models, metabolic availability and toxicity. *Environmental International*, 33: 576-582.

Ramalhosa, E., Monterroso, P., Abreu, S., Pereira, E., and Duarte, A. (2001). Storage and export of mercury from a contaminated Bay (Ria de Aveiro, Portugal). *Wetlands Ecology and Management*, 9: 311-316.

Ramalhosa, E., Pereira, E., Vale, C., Válega, M., Duarte, A.C. (2005). Distribution of mercury in the upper sediments from a polluted area (Ria de Aveiro, Portugal). *Marine Pollution Bulletin,* 50: 682-697.

Ramalhosa, E., Pato, P., Monterroso, P., Pereira, E., Vale, C., Duarte, A.C. (2006). Accumulation versus remobilization of mercury in the sediments of a contaminated lagoon. *Marine Pollution Bulletin*, 52,:353-356.

Reis, A.T., Rodrigues, S.M., Araújo, C., Coelho, J.P., Pereira, E., Duarte, A.C. (2009). Mercury contamination in the vicinity of a chlor-alkali plant and potential risks to local population. *Science of the Total Environment,* 407: 2689-2700.

Richard, A. (1971). Contribution à l'étude expérimentale de la croissance et de la maturation sexuelle de *Sepia officinalis* L. (Mollusque: Céphalopode). Thèse de Doctorat d'État, Université de Lille, 243 : 1-2264.

Rodhouse, P.G. and Nigmatullin, Ch. M. (1996). Role as consumer. *Philosophical Transactions of the Royal Society of London.* B 351(1343), 1003-1022.

Roesijadi, G. (1993). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology*, 22: 81–114.

Roesijadi, G. and Robidon, W. (1994). Metal Regulation in Aquatic Animals: Mechanisms of Uptake, Accumulation and release. In: Aquatic Toxicology – Molecular, Biochemical and Cellular Perspectives. Ed. Mallins, D. and Ostrander, G., Lewis Publishers, CRC-Press EUA, 387-420.

Roper, C. F. E., Sweeney, C.E., Nauen, C.E. (1984). FAO Species catalogue. Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. *FAO Fisheries Synopsis*, FAO Fish, 125(3),: 277.

Serrano, M. (1992). Contribuição para o conhecimento da biologia e pescaria do choco, *Sepia officinalis* (Linnaeus, 1758) no estuário do Sado e zona costeira adjacente. Relatório Técnico-Científico, INIP, Lisboa.

Shi, D. and Wang, W. –X. (2005). Uptake of aqueous and dietary metals by mussel *Perna viridis* with different Cd exposure histories. *Environmental Science & Technology*, 39: 9363-9369.

Silva, J.N. (2000). *Aveiro Natural*. Livros Quetzal, Aveiro.

Simkiss, K. and Taylor, M.G. (1995). Transport of metals across membranes. *In:* Tessier, A., Turner, D.R. (Eds.), Metal Speciation and Bioavailability in Aquatic Systems. Wiley, Chichester, pp. 2–44.

Sinanoglou, V. J. and Miniadis-Meimaroglou, S. (1998). Fatty acid of neutral and polar lipids of (edible) Mediterranean cephalopods. *Food Research International*, 31: 467–473.

Sinanoglou, V. J., and Miniadis-Meimaroglou, S. (2000). Phospholipids in Mediterranean cephalopods. *Journal of Bioscience*, 55: 245–255.

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I. (2010). The influence of *Spartina maritima* on carbon retention capacity in salt marshes from warm-temperate estuaries. *Marine Pollution Bulletin*, 61, 215-223.

Sunda, W.G., Huntsman, S.A. (1998). Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems. *Science of the Total Environment*, 219(2-3), 165-181.

Sweeney, M. J., Roper, C. F. E. (1998). Classification, type localities, and type repositories of recent cephalopoda. *Smithsonian Contributions to Zoology*, 0(586 Part 2): 561-595.

Sykes, A., Domingues, P., Correia, M., Andrade, P., (2006). Cuttlefish culture: state of the art and future trends. *Vie et Millieu*, 56: 129–137.

Sykes, A.V., Oliveira, A.R., Domingues, P.M., Cardoso, C.M., Andrade, J.P., Nunes, M.L. (2009). Assessment of European cuttlefish (*Sepia officinalis*, L.) nutritional value and freshness under ice storage using a developed Quality Index Method (QIM) and biochemical methods. *LWT - Food Science and Technology*, 42, (1), 424–432.

Turner, D.R. and Tessier, A. (1995). Metal Speciation and Bioavailability in Aquatic Systems. Wiley, Chichester.

Ullrich, S., Ilyushchenko, M., Kamberov, I., Tanton, T. (2007). Mercury contamination in the vicinity of a derelict chlor-alkali plant. Part I: sediment and water contamination of Lake Balkyldak and the River Irtysh. *Science of the Total Environment*, 381: 1–16

UN Atlas of the Oceans, (2010). Available at: http://www.oceansatlas.org/servlet/CDSServlet?status=ND0xODc3JjY9ZW4mMzM9KiYzNz1rb3M~

UNEP (1997). Report of the meeting of experts to review the MEDPOL biomonitoring programme. Athens, Greece: UNEP-(OCA)/MED WG, 132-137.

UNEP (2004). Guidance for a Global Monitoring Programme for Persistent Organic Pollutants. Inter-Organization Programme for the Sound Management of Chemicals. Geneva, Switzerland.

Válega, M., Abreu, S., Pato, P., Rocha, L., Gomes, A.R., Pereira, M.E., Duarte, C. (2006). Determination of organic mercury in biota, plants and contaminated sediments using a termal atomic absorption spectrometry technique. *Air, and Soil Pollution*, 174: 223-234.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13(2): 57-149.

Vasconcelos, R.P., Reis-Santos, P., Fonseca, V., Maia, A., Ruano, M., França, S., Vinagre, C., Costa, M.J., Cabral, H.N. (2007). Assessing anthropogenic pressures on estuarine fish nurseries along the Portuguese coast: a multi-metric index and conceptual approach. *Science of the Total Environment*, 374: 199–215.

Viarengo, A., Blasco, J., Fabri, R., Burlando, B., Ponzano, E., Marchi, B., Trielli, F. (1998). Metallothionein and oxidative stress in marine organisms. *Marine Environmental Research*, 46(1-5): 606-607.

Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W.J.G.M. (2004). Internal metal sequestration and its ecotoxicological relevance: A review. *Environmental Science & Technology*, 38(18): 4705-4712.

Wallace, W.G., Lee, BG., Luoma, S.N. (2003). Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Marine Ecology Progress Series*, 249: 183–197.

Wang, W-X. (2002). Interactions of trace metals and different marine food chains. *Marine Ecology Progress series*, 243: 295–309.

Wang, W.-X. and Rainbow, P.S. (2005). Influence of metal exposure history on trace metal uptake and accumulation by marine invertebrates. *Ecotoxicology and Environmental Safety* , 61, 145–159.

Wang, W.-X. and Rainbow, P.S. (2008). Comparative approaches to understand metal bioaccumulation in aquatic animals. *Comparative Biochemistry and Physiology*, Part C 148: 315–323.

Ward, P.D., Boletzky, S.V. (1984). Shell implosion depth and implosion morphologies in the three species of *Sepia officinalis* (Cephalopoda) from the Mediterranean Sea. *Journal of Marine Biology Association*, UK, 64: 955-966.

Wittman, G.T.W. (1979). Toxic metals. In: Forstner, U., Wittmann, G.T.W. (Eds.), Metal Pollution in the Aquatic Environment. Springer Verlag, Berlin, pp. 3-70.

Xu, Y. and Wang, W.-X., (2002). Exposure and potential food chain transfer factor of Cd, Se and Zn in marine fish Lutjanus argentimaculatus. Marine Ecology Progress series, 238: 173–186.

Zhang, L. and Wang, W.-X. (2006a). Alteration of dissolved cadmium and zinc uptake kinetics by metal pre-exposure in the black sea bream (*Acanthopagrus schlegeli*). *Environmental, Toxicology and Chemistry*. 25: 1312–1321.

Zhang, L. and Wang, W.-X., (2006b). Significance of subcellular metal distribution in prey in influencing the trophic transfer of metals in a marine fish. *Limnology and. Oceanography*. 51: 2008–2017.

Zlatanos, S., Laskaridis, K., Feist, C., and Sagredos, A. (2006). Proximate composition, fatty acid analysis and protein digestibility-corrected amino acid score of three Mediterranean cephalopods. *Molecular Nutritonal Food Research*, 50: 967–970.

CHAPTER II

Ecology and life cycle of common cuttlefish *Sepia officinalis* in a typical estuarine lagoon

II. Ecology and life cycle of common cuttlefish *Sepia officinalis* in a typical estuarine lagoon

II.1.Abstract

The cuttlefish (*Sepia officinalis*) is one of the most important fisheries in Ria de Aveiro. Temporal and spatial distribution patterns and the reproductive cycle of this specie were studied monthly in ten sampling areas along the lagoon from July 2001 to December 2003. The highest abundance was obtained in summer, while spring showed the higher biomass but a much lower abundance of specimens. Mature individuals prevailed in the eastern zone of the lagoon during summer while juveniles were dominant in autumn and winter. Almost all individuals leave the lagoon during winter. The reproduction season began in spring and continued during summer, the data did not reveal the existence of a second reproductive cycle in the autumn and winter. Weight–length relationship revealed significant differences between sexes, for lengths higher than 82.4 mm females were larger than males. A sex ratio of 1:1 was presented in this studied. The length at first maturity was estimated at 146.9 ± 0.8 mm dorsal mantle length (DML) for females and 91.4 ± 0.9 mm DML for males, and it was verified that males mature earlier than females. In Ria de Aveiro only one generation of cuttlefish breeders were found and all the individuals were in their first year of life. In terms of trophic ecology, the cuttlefish is an opportunistic feeder, consuming several preys of different taxa. Diet was not different between selected sites, however reveled differences between seasons and preys. The preferred prey for *Sepia officinalis* were the group of Portunidae, and the species *Crangon crangon*, *Carcinus maenas* and *Pomatoschistus microps* were secondary preys.

II.2.Introduction

Like other cephalopods, *Sepia officinalis* reproduces only once over a short period at the end of its life (Mangold, 1983). The main stages of its life are punctuated by important seasonal migrations between shallow waters in summer and deeper waters in the winter (Gauvrit *et al.*, 1997). Cuttlefish live approximately two years and exhibits mass mortality of adults following spring spawning period (Boletzky, 1983). Although cuttlefish can exhibit variations in the life cycle, *e.g.* Domingues *et al.* (2002) referred that some specimens can only reach 6 to 9 months of age. The duration of its life cycle is closely related to environmental factors, like the temperature of water (Domingues *et al.*, 2002). Thus, in the northern part of their distribution area (English Channel), they reproduce during the second year of life, during a short breeding season of 2 to 3 months (Boucaud-Camou *et al.*, 1991). In contrast, in Warmer waters such as those around the Iberian Peninsula, the Mediterranean and the Gulf of Tunis, the majority of cuttlefish reproduce at one year old and over a longer period (Coelho and Martins, 1991; Guerra and Castro, 1988; Mangold, 1966).

Due to the seasonal migrations for reproduction, the cuttlefish is exposed to a wide range of diets (Guerra, 2006). Several authors have studied the feeding habits of cuttlefish in northern Spain, France and Mediterranean (Najai and Ktari, 1979; Castro and Guerra, 1989, 1990; Pinczon du Sel and Daguzan, 1992; Blanc, 1998; Pinczon du Sel *et al.*, 2000; Boucaud-Camou *et al.*, 1985). However, in Portugal studies about diet only appears in south coast, Alves *et al.*, (2006) in Algarve and Neves *et al.*, (2009) in Sado estuary. In the northern of Portugal, none published data about feeding habits in known.

Cephalopods have growth rates that can be higher than 10% body weight day^{-1} (Domingues *et al.*, 2001b). Since cephalopods are poikilothermic, temperature is the most important factor affecting growth rates when food is abundant (Forsythe and Van Heukelen, 1987). Beyond the duration of life cycle and growth rates of cuttlefish, many others characteristics of their life cycle are affected by temperature such as survival and feeding rates, age and size maturation, embryonic development and hatchling survival (Boletzky, 1979; Boletzky and Hanlon, 1983).

Sepia officinalis L., is one of the best known Cephalopods (Guerra and Castro, 1988). Several studies have been conducted on distribution (Gi Geon 1981; Boletzky, 1983; Roper *et al.*, 1984; Kromov *et al.*, 1998; Nixon and Mangold, 1998), biology (Ezzedine-

Najal S. 1984; Guerra, 1992; Clarke, 1996; Norman, 2000), life cycle (Choe, 1966; Boletzky, 1979; Boletzky, 1983; Forsythe *et al.*, 1994; Le Goeff and Daguzan, 1991; Bouchaud, 1991; Nixon and Mangold, 1998; Önsoy B. and Salman A. 2005), maturation (Bouchaud and Daguzan, 1989; Richard, 1971), aquaculture (Hanley *et al.*, 1998; Pascual, 1978), diet (Pinczon du Sel and Daguzan, 1992; Blanc *et al.*, 1999; Koueta and Boucaud-Camou, 1999; Domingues *et al.*, 2001a; Koueta *et al.*,2002) and external factors (Palmegiano and D'apote, 1983; Bouchaud and Daguzan, 1990; Domingues *et al.*, 2001b). However, in spite of its economic importance in the Ria de Aveiro (Docapesca - Statistical Department, 2013), this species has not yet received consistent scientific attention, the information is scarce and only one study by Jorge and Sobral (2004) gave some information about species biology in Ria de Aveiro.

The aim of this research is to improve the knowledge on the population structure of *Sepia officinalis* in Ria de Aveiro (abundance, biomass (total weight), length, distribution of sex and maturity stages); to describe the diet of *Sepia officinalis* in Ria de Aveiro and the reproductive cycle of *S. officinalis*, in Ria de Aveiro lagoon.

II.3.Materials and methods

II.3.1. Study Area

Ria de Aveiro is a coastal lagoon (Figure II.1), also mentioned by others author as a ''bar-built estuary'' (Dias, 2001). It is located in the northwestern (40º38´N, 8º44´W) coast of Portugal. The system can be subdivided into channels that, due to specific characteristics, can be regarded as independent estuaries connected to a common outlet. In general, the

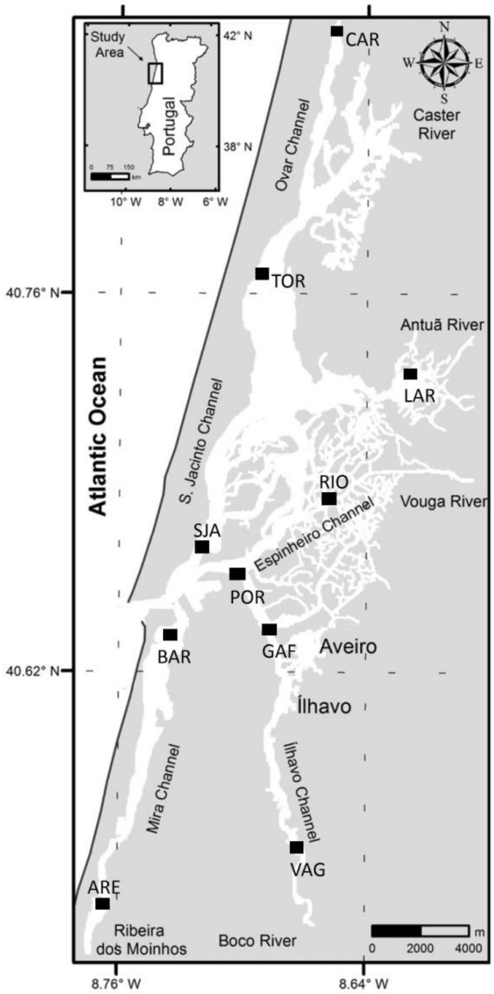

Figure II.1 - Sampling sites of *S. officinalis*: Ria de Aveiro (Adapted from Valentim *et al.*, 2013).

most common typology is one of extensive intertidal zones, namely mud flats and salt marshes, with the exception of the central area of the lagoon (Pastorinho *et al*., 2012). Sampling sites were selected based on a geographic distribution taking into account the whole area of the lagoon. ARE, at the end of Mira channel (the Southern site); BAR near the entrance of the lagoon; SJA near the entrance of the lagoon in the initial part of S. Jacinto channel; POR within the harbour; GAF situated in the vicinity of a deep-sea fishing port and dry-docks; TOR an intermediate region of the longest channel (S. Jacinto-Ovar channel); RIO located in the terminal area of the main freshwater course, the Vouga river; LAR in the proximity of the chlor-alkali plant (6 km); CAR located in the terminal area of the S. Jacinto channel (the Northern site) and VAG located at the edge of Íllhavo channel.

II.3.2. Sampling and biological data collection

Sampling was made monthly, from July 2001 to December 2002 with bottom trawl and from March 2003 to December 2003 with a trammel network (Table II-I). In the years of 2001 and 2002, samples were collected in 10 sites of Ria de Aveiro (Figure II.1). The reason to use two different types of networks was the necessity of capturing cuttlefishes of all maturity stages, to have a representative structure of the population. A total of 295 cuttlefishes were captured.

II.3.3. Abiotic parameters

The abiotic parameters such as temperature, dissolved oxygen, pH, salinity, turbidity and depth were registered on each capture. Temperature (± 0.1 ºC) and dissolved oxygen (± 0.01 $mg.l^{-1}$) were recorded with an oxygen meter (Consort Z621), the pH (± 0.01) with pH meter (*WTW* 330/set – 2), the salinity (± 0.1) with a refractometer (*Atago*), the turbidity (± 0.1 m) was recorded with a Secchi disc and the depth (± 0.1m) with a handmade probe (Table A. 1 - Appendix).

II.3.4. Biological parameters

Dorsal mantle length (DML, size), total weight (W_t), the wet weight of the gonad (WG), and for females the wet weight of the nidamental glands, the sex and a maturity stage were determined in each individual. The number of individuals, mantle length and body weight of each site are given in Table II-I.

To determine the relationship between DML and Wt for males and females the parameters of the equation Wt = a DML^b were estimated. A standard 5-point scale of sexual maturity for *S. officinalis* was used (adapted from Alonso-Allende and Guerra, 1984), in which stage I is “immature”, stage II is “developing”, stage III is ”maturing”, and stage IV is “ripe”. Specimens with no visible gonad development were classified as “juveniles” (Challier *et al.*, 2002) and allocated in maturity stage I.

II.3.5. Trophic ecology parameters

The analysis of gut contents was made with use of binocular lens, by separating prey by their different taxa, and being the identification of their taxonomic level made according to the digestion level.

The prey´s identification was not made with the aim of reaching their specie, because it was considered that such precision was not necessary, and they were only identified to the lower taxonomic category possible. Other studies also suggest that it is not necessary to reach the species according to the proposed objectives (Windell and Bowen, 1978; Oliveira, 1997). Reaching the specie identification is a complex and time consuming task that would not add significant information.

The identified preys were counted by taxa for each gut. The counting process was a difficult lengthy operation, as they were frequently disarticulated, and therefore it was decided to choose fragments that existed individually on each prey of a certain tax, as they might be considered diagnostic features.

The unidentified material was classified as detritus, and counted as a single item, by each structure were it was identified as proposed by Madenjian *et al*. (1996). The different types of algae and marine angiospermae found, independently of the number of fragments, were counted as a unique feed component, being inserted as Vegetable.

After their identification and counting by taxa, preys were preserved in 70% alcohol (ethanol) (Windell and Bowen, 1978).

Wet weight (WWt) was determined for the different preys by taxonomic group in a 4 decimal places scale. Several authors use WWt instead of the dry weight (DWt), which is the most accurate measure to use in the weight coefficient (C_w %) (Hynes, 1950; Windell and Bowen, 1978) and it is known that both are strongly correlated (Glenn and

Ward, 1968). Prior to the identification ash-free dry weight (AFDW) was obtained after 24h in an oven at 60ºC.

Detritus were not used on later analysis, as performed by other authors (Caragitsou and Papaconstatinou, 1994; Capitolo *et al*., 1995).

All biologic characteristics obtained from the monthly sampling campaigns were recorded as well as the ash-free dry weight (AFDW).

II.3.6. Data analysis

II.3.6.1. Abiotic data analysis

Principal Component analysis (PCA) was performed, using the CANOCO software package for Windows, version 4.5 (Ter Braak and Smilauer, 2002) to assess the variability of abiotic parameters measured during the study period (2001-2003) in the different sampling sites.

II.3.6.2. Biological data analysis

The length at which 50% of cuttlefish were mature was determined for females and males. The percentage of sexually mature individuals (PM), for each sex (maturity stages III and IV) caught during sampling were determined by fitting the following logistic model:

$PM = \frac{DML^i}{DML^i + SFM^i}$, where SFM is the size at first maturation.

An index of sexual development was calculated using the gonadosomatic index (GSI), which is defined as

$GSI = \frac{GW}{(Wt - GW)} \times 100$, was estimated for season and maturity stage.

The sex-ratio was calculated for each season. Seasons were defined as follow:

Winter = January – March. Spring = April – June

Summer = July – September. Autumn = October – December.

The comparison of allometric relationships between males and females were tested using ANCOVA (Zar, 1999). Redundancy Analysis (RDA), a technique that falls between multiple regression and canonical correlation analysis (Sparks *et al*., 1999), was performed using the CANOCO software package for windows, version 4.5 to assess effects of biological factors on *S. officinalis* in the three years of sampling and in sites sampling. The significance of RDA was tested using a Monte Carlo permutation test using routines built into the software package. A complete description of the method is provided in Van den Brink *et al.* (2003). Differences on the gonadosomatic index (GSI) for seasons and sites were evaluated using the Kruskal-Wallis test using MINITAB® Release 14.20. A significance level of 0.05 was used for all tests. The deviations from 1:1 sex ratio were assessed using chi-square test by Sigmaplot 10.

II.3.6.3. Diet analysis

Gut content analysis method

To analyse the gut contents three methods were used:

Numeric method;

Occurrence (or frequency) method;

Gravimetric (or volumetric) method.

These methods can be complemented by other "mixed" methods (data interaction between two or more of the base methods referred), as well as indexes and additional information that could help on the diet characterization.

They produce accurate results, both at individual and conjugated level.

Numerical method (C_N %)

The total number of individuals of a certain taxa is expresses as a percentage of the total number of preys (Hynes, 1950; Pillay, 1952; Hyslop, 1980; Bowen, 1983; Valente, 1992):

$$C_N = \frac{n}{N} * 100$$

Where n – total number of preys of a certain taxa; N – total number of preys.

Problems associated with this method and their specific resolution was previously referred in Materials and methods.

Gravimetric method (C_w %)

The weight estimation of each taxa (in fresh or dry) is calculated as a percentage of the total weight of the contents (Pillay, 1952; Hyslop, 1980; Bowen, 1983):

$$C_w = \frac{w}{W} * 100$$

Where w – total wet weight of the preys of a certain taxa; W – Wet weight of all preys.

Gravimetric method gives a reasonable estimate of the preys weight, being easy to apply when residues are high (Valente, 1992) overvaluing the individual contribution of heavy taxa (George and Hadley, 1979).

Occurrence method

In this method, the number of individuals in which a determined item occurs is expressed in terms of frequency of the total number of guts sampled (Hynes, 1950; Pillay, 1952, 1983; Hyslop, 1980; Valente, 1992):

$$C_F = \frac{f}{F} * 100$$

Where f – number of guts where a certain taxa is present; F – Total number of full guts observed.

This method can be used as an interspecific competition indicator. If C_F% exceeds 25% in two or more predators, competition is possible (Hyslop, 1980). It is also considered useful on the determination of seasonal variations of diet composition (Frost, 1977; Pedley and Jones, 1978), as food selectivity by the predator and as an availability index of a certain food (Herrán, 1988).

Feed coefficient (Q)

The feed coefficient, by combining more than one method, is considered a mixed method (Herrán, 1988). Thus it allows a good analysis of the relative importance of different preys of a certain animal, by taking into account their contribution in weight and number (Hureau, 1970):

$$Q = C_N\,\% * C_P\,\%$$

Depending on the Q value, three categories were defined (Hureau, 1970):

Q<20 – Occasional preys;

20<Q<200 – Secondary preys;

Q>200 – Preferential preys.

Statistical analysis

Differences in AFDW of gut contents between sampling sites and the food types were assessed using the GLM module of Minitab 16 (Minitab Inc., 2010). Prior to the analysis the data was check for normality and homoscedasticity using the Anderson Darling Test and the Levene test, respectively. To correct for deviations to normality and heteroscedasticity AFDW was transformed using the log(X+1) transformation prior to further analysis. Specific size differences associated with the diet were assessed using the eviscerated weight of the individuals as a covariate. Whenever significant differences associated with a given factor were found the Tukey post hoc test was applied to identify differences between levels. All statistical analysis was carried out for a significance level of 0.05.

Table II-I – Summary of key aspects related with the structure of cuttlefish populations (number of individuals (n), mantle length (mm) and total weight (g)) by year of capture.

Sites	n			Mantle lenght (mm)			Total weight (g)		
	2001	2002	2003	2001	2002	2003	2001	2002	2003
BAR	11	19	12	18.8 - 107.0	20.0 - 112.2	32.0 - 187.0	170.0	797.5	3170.4
CAR	17	6	-	30.2 - 67.3	25.8 - 110.0	-	237.0	204.7	-
GAF	14	34	5	31.1 - 67.2	15.3 - 172.0	91.0 - 187.0	322.0	1482.6	1497.8
LAR	28	16	3	23.6 - 129.0	32.8 - 144.1	140.0 - 185.0	821.6	1177.5	1437.3
RIO	19	11	3	15.6 - 148.6	16.0 - 106.4	108.0 - 113.0	688.8	246.5	586.0
SJA	10	12	-	17.1 - 57.4	17.0 - 78.0	-	64.4	337.7	-
TOR	19	16	8	26.7 - 115.6	35.8 - 85.6	105.0 - 185.0	434.6	444.2	3494.4
POR	-	-	25	-	-	95.0 - 208.0	-	-	14095.8
VAG	3	4	-	32.1 - 53.2	39.9 - 125.4	-	49.2	395.3	-
ARE	-	-	-	-	-	-	-	-	-
TOTAL	121	118	56	15.6 - 148.6	15.3 - 172.0	32.0 - 208.0	2787.6	5086.0	24281.7

II.4.Results

II.4.1. Abiotic parameters

PCA analysis showed that most of the variability is associated with the first two axes (76.1%), and the rest of the axis only explained 23.9%. The PCA analysis of abiotic parameters within different years of capture in Ria de Aveiro (Figure II.2; a)) was made assuming the existence of annual variation. However, the analysis showed that the differences in abiotic parameters didn’t lead to differences in between years. A good overlap of the three years of sampling was observed (Figure II.2; a)). Having this fact in mind, the analysis of three years of catches can be done together and the variation of abiotic parameters was not related to months but to sites of sampling (Figure II.2; b)). The PCA analysis of abiotic parameters within different sites of capture in Ria de Aveiro (Figure II.2; b)) showed that the main two factors that affected *S. officinalis* were salinity and depths (Figure II.2; a) and b)). Indeed the only site where the cuttlefish was not caught was Areão (ARE), which was the site that presented the lowest values of salinity and depth (Figure II.3 and Figure II.4). ARE presented salinity values between 3 and 26, being the mean value 11 ± 6 (average ± SD) and depths of 1.21 ± 0.35 m (average ± SD). Vagos (VAG) presented wide–range of salinity amplitudes that also influenced the number of cuttlefishes captured (Table II-I). VAG presented salinity values between 1 and 37, being the mean value 21 ± 9 (average ± SD). These two sampling sites, ARE and VAG, are included in the dashed ellipsoid area of the PCA biplot (Figure II.2; b)) and were sites where the sampling method was only the bottom trawl, because they had no adequate conditions for the trammel network.

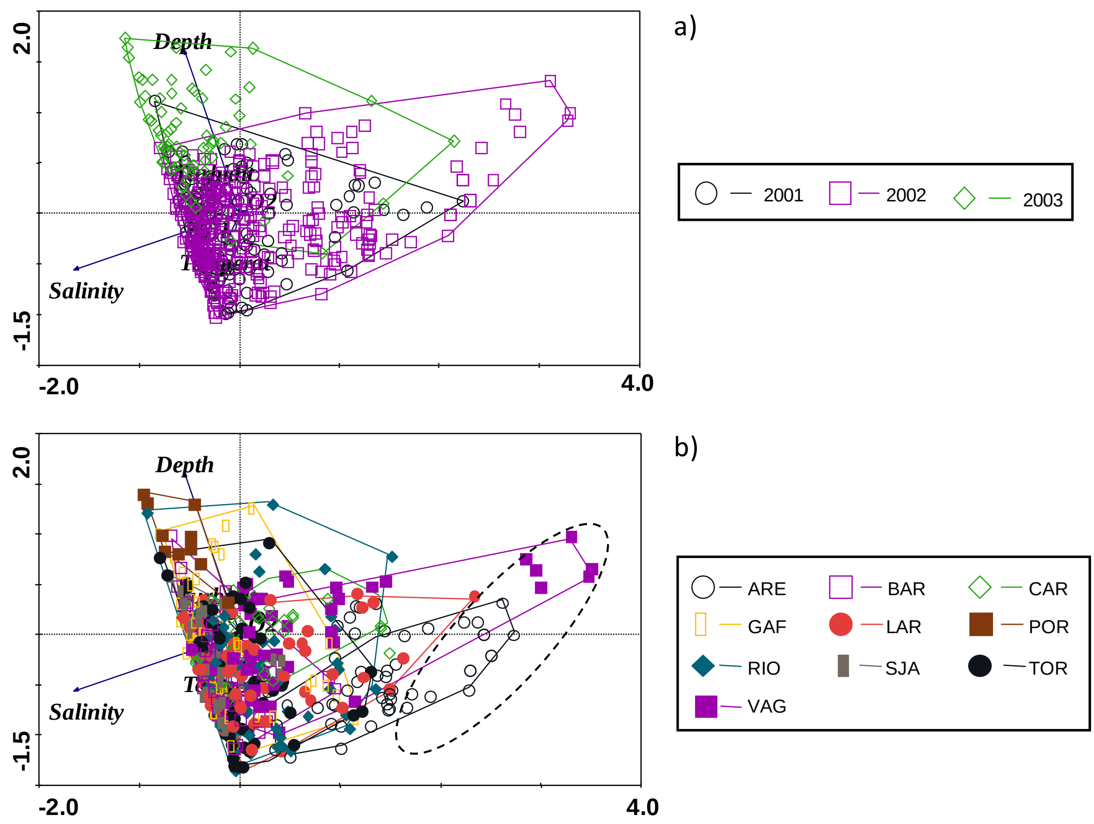

Figure II.2 – Biplot for the PCA analysis of physical and chemical parameters measured during the sampling campaign grouped by a) sampling year and b) sampling site. The dashed ellipsoid delimits observations made in sites sampled exclusively with bottom trawl in 2001-2002.

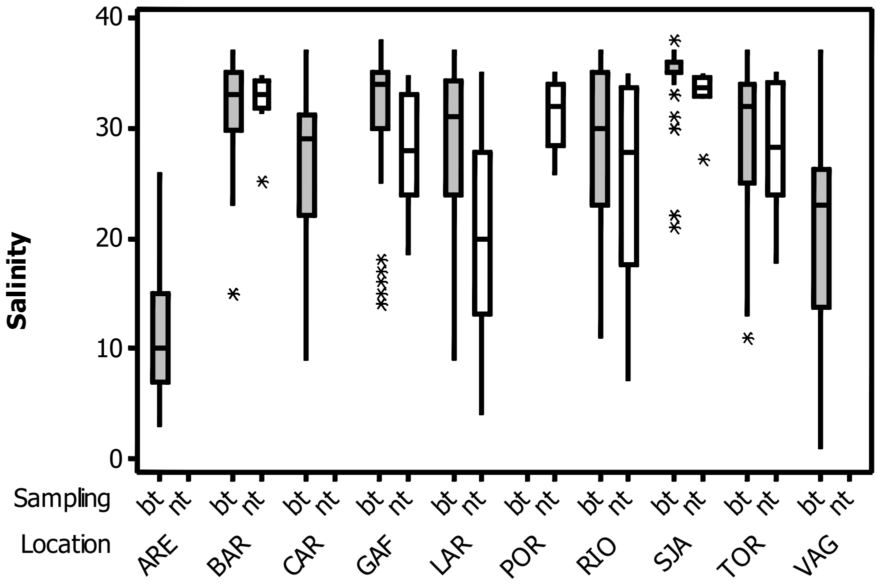

Figure II.3 – Variation in salinity measured during the campaigns with different sampling techniques of cuttlefish (bt – bottom trawl and nt – network trammel) by site sampling.

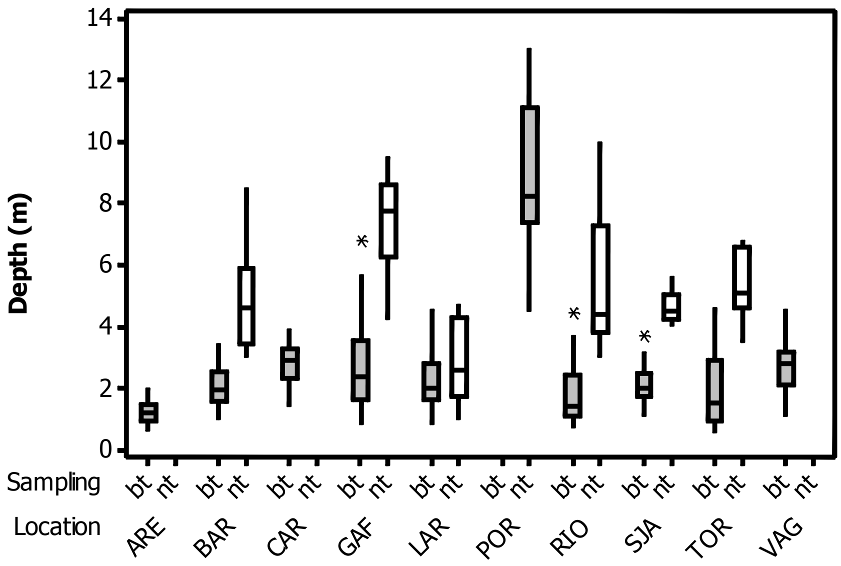

Figure II.4 – Variation in depth (m) measured during the campaigns with different sampling techniques of cuttlefish (bt – bottom trawl and nt – network trammel) by site sampling.

Boxplot analysis allowed a more comprehensive view of salinity and depths variations (Figure II.3 and Figure II.4). Commercial port (POR) was the station that most contributed to the global biomass of *S. officinalis* captured (Table II-I and Figure II.7) and had the higher value of depth (Figure II.4). By crossing the depth values obtained with the individual captured on each site (Table II-I), it is possible to state that cuttlefish prefers higher depths, what may also justify the inexistence of individuals caught on Areão.

II.4.2. Weight-length relationship

Both males and females showed allometric growth (Figure II.5), and the analysis of covariance (ANCOVA) of log transformed length and weight data showed a significant difference between the growth pattern of males and females ($p<0.05$).

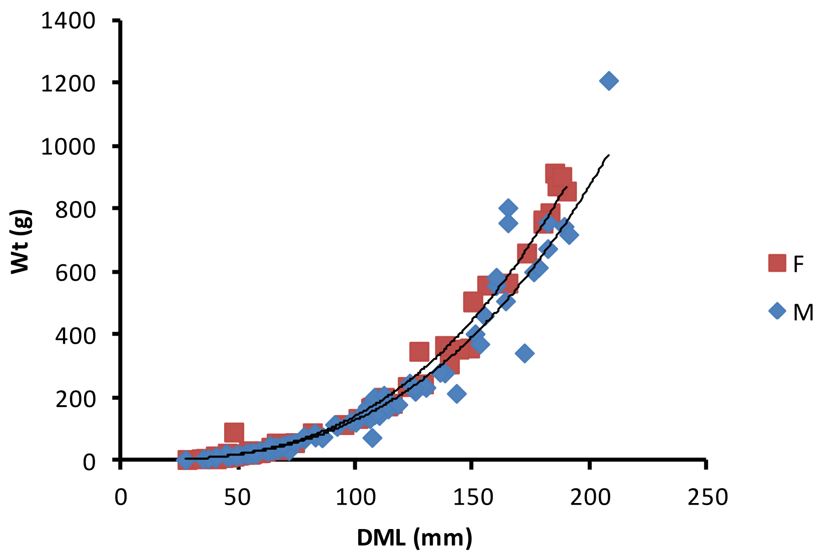

Figure II.5 – Length-weight relationship of female (red squares) and male (blue rhombus) of the *S. officinalis* captured in the Ria de Aveiro.

Regression lines were not coincident (ANCOVA: $F_{2,\ 148}= 3.154$; $p=0.046$). Slopes did not differ significantly ($t_{148}=0.974$; $p=0.332$), but intercepts differed significantly ($t_{148}=2.315$; $p=0.022$). For both sex the values obtained for slope (b) was lower than 3, corresponding to a negative allometry ($b<3$), confirming a higher increase of length compared to weight. Although the slope (b) of the two curves was not significantly different (Table II-II), the female regression presented a higher slope when compared to the male regression. Smaller individuals have a similar total weight, but for individuals greater than 82.4 mm, females were heavier than males (Figure II.5).

Table II-II – Parameters of the length-weight relationship of *S. officinalis* according to sex.

Sex	a	b	r^2	N
Female	0.2014	2.8343	0.9728	64
Male	0.2144	2.7741	0.9811	88

II.4.3. Distribution patterns

From the 295 cuttlefishes analysed: 89 were females, ranging from 28 mm to 190 mm; 105 were male, ranging from 27 mm to 208 mm; and 143 were indeterminate, from 17 mm to 67.3 mm ML. Figure II.6 shows that the frequency of individuals caught by mantle length class, sampled with the same network (2001 and 2002), present a similar structure distribution. This result was also verified with the RDA analysis along the years of sampling, which presented no significant differences for sex, sites and maturity stages ($p>0.05$). In fact, when DML (mm) was removed from the analysis, the RDA revealed no significant difference in the *S. officinalis* distribution between sex and maturity stages ($p>0.05$), indicating that the difference was a consequence of network caught.

The highest abundance was registered in summer (225 individuals) and the lowest abundance was registered in winter (only, 3 individuals) (Figure II.7). The central part of the lagoon (near the mouth) was the most abundant in cuttlefish, while the most Northerly and Southerly sites presented the lowest abundance in this species. This fact is related to abiotic parameters (Figure II.2, b)), since sampling sites located within the limits of the Ria de Aveiro showed the lowest values of salinity. The highest biomass values were recorded in spring and summer (13 and 18 Kg, respectively) and the highest value by sampling site was obtained in POR (14.1 Kg) (Figure II.7).

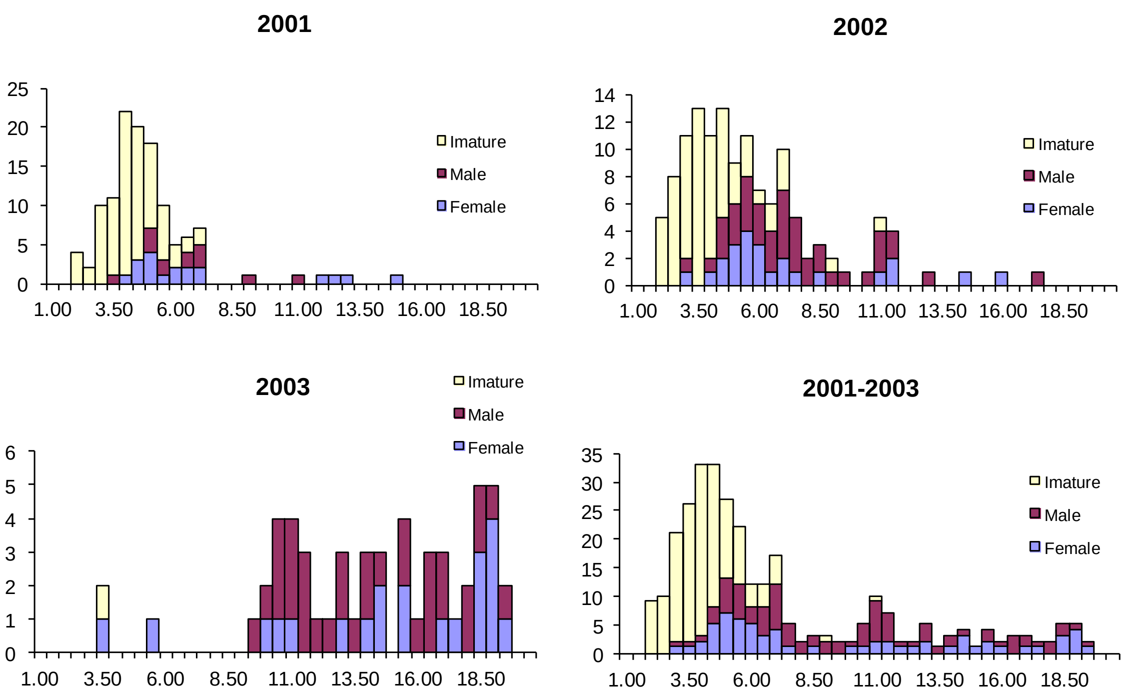

Figure II.6 – Length frequency distribution of immature, male and female cuttlefish along years of sampling.

The abiotic parameters provide the colonization of the lagoon by cuttlefish practically throughout all the year, although with marked fluctuations in abundance. During summer the cuttlefish appears with more frequency in the lagoon (Figure II.7), and the cuttlefish population is comprised by males, females and immature individuals (Figure II.6 and Figure II.8). In terms of sexual maturation, the dominant stage of cuttlefish observed was predominantly the immature stage of gonadal development, which reinforces the young character of cuttlefish population in the lagoon (Figure II.6 and Figure II.8).

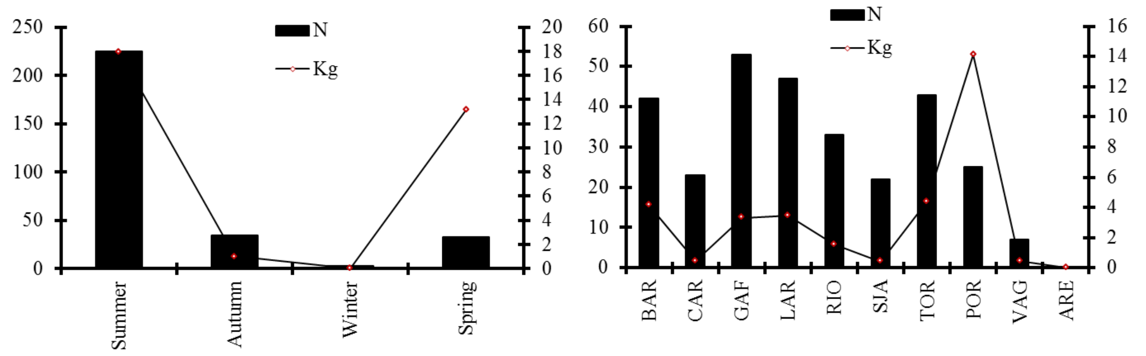

Figure II.7 - Number of individuals (N) and total weight (Kg) for seasons and sites sampling, for the three years of sampling.

Cuttlefish captured in Ria de Aveiro belonging to first maturity stage (I) presented DML between 15.3 to 73.9 mm; developing maturity stage (II) presented values between 44.6 and 115.6 mm; maturing stage (III) ranged from 82.7 to 185.0 mm and ripe cuttlefish (IV) ranged between 78.0 and 208.0 mm. The analysis of the percentage of individuals per maturity stage along the three consecutive years of sampling (Figure II.8) showed that: large cuttlefish, higher than 82.7 mm, enter the Ria in spring; during summer and autumn the smaller individuals (stage I and II) dominate, however the expression of mature individuals in summer is still very high; in winter the abundance of *S. officinalis* in the Ria is strongly reduced. This fact suggests that the development of the cuttlefish born in Ria de Aveiro is mainly made offshore, what could justify the absence of maturing individuals during the autumn (Figure II.8). The absence of mature specimens after the spawning season (spring and summer) suggests that cuttlefish does not survive after spawning (Figure II.8). The spawning occurs in the late spring and early summer. With the exception of Carregal (CAR), in all other sites of Ria de Aveiro, ripe individuals were present (Figure II.8). However, by making a seasonal analysis, it was possible to observe that mature individuals were only captured in spring and summer (Figure II.8).

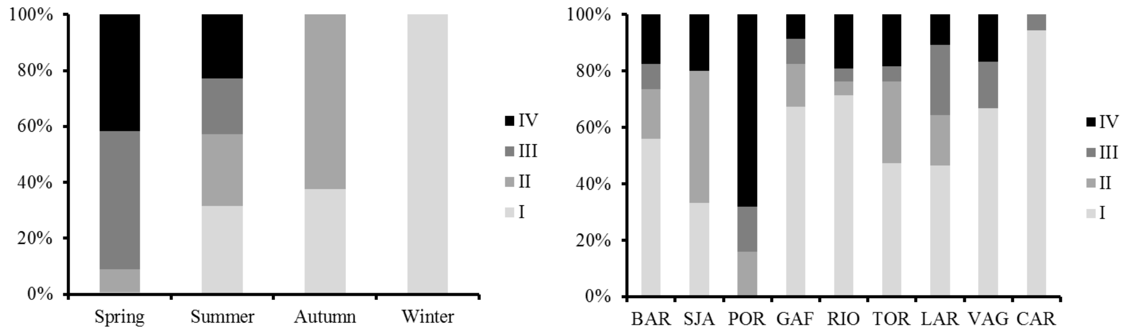

Figure II.8 – Percentage of individuals per maturity stage (I, II, III and IV) throughout the seasonal and spatial variation, along the Ria de Aveiro.

II.4.4. Reproduction strategies

As stated above the cuttlefish enters the Ria de Aveiro in spring and summer for reproduction, and returns offshore with winter. Immature females ranged between 28 mm and 73.9 mm, and developing specimens ranged between 45.2 mm and 114.6 mm. Maturing females were caught with mantle length from 122.1mm to 185.0 mm, and females in ripe conditions varied from 106.4 mm to 190.0 mm. Immature males were found from 27.3 mm to 106.9 mm, while specimens in developing stage ranged between 44.6 mm and 109 mm. Maturing males were caught with mantle length ranged from 82.7 to 130 mm, and males in ripe conditions varied from 78 mm to 208 mm. From the 295 cuttlefishes caught in Ria de Aveiro, 89 were females and 105 were males, significant differences in the GSI values were obtained between seasons for both females ($H = 29.6$, $P<0.001$) and males ($H = 54.9$, $P<0.001$). Females showed high GSI values from April to July, noticing greater increase from April to June and a marked decrease from June to July (Figure II.9). The females caught in June had the higher GSI of the study period, and the principal maturity stage of the females in that month belongs to the third maturity stage (III), the developing stage. A similar pattern was noticed for males, with higher GSI index in June, although these values were considerably lower than those registered for females (Figure II.9). Significant differences in the GSI values were obtained between maturity stages for both females ($H = 52.2$, $P<0.001$) and males ($H = 73.2$, $P<0.001$). GSI increased according to maturity stage in females and males (Figure II.9), although a slight decrease was observed from stage III to stage IV, for both sexes.

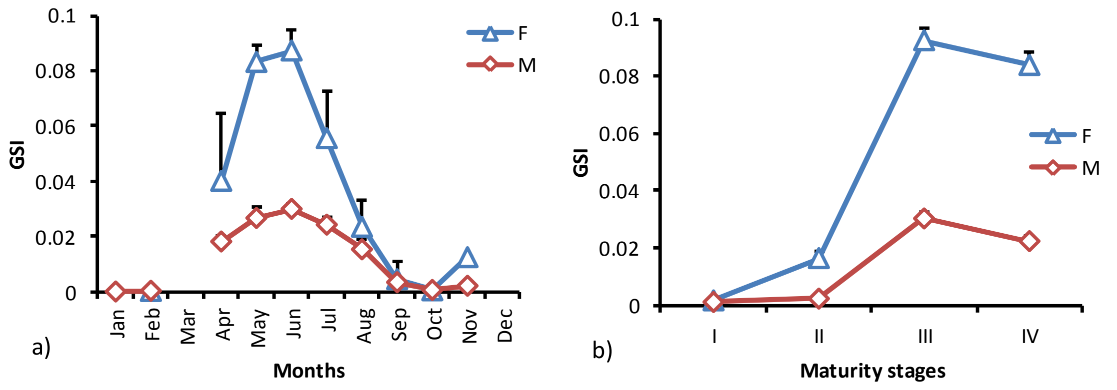

Figure II.9 – Gonadosomatic index (GSI) values for males (M; N = 105) and females (F; N = 89) of *S. officinalis* throughout the months sampling [a)] and by maturity stages [b)].

Length at first maturity was estimated as 146.9 ± 0.8 mm DML for females and 91.4 ± 0.9 mm DML for males (Figure II.10). A total of 89 females and 105 males were caught in Ria de Aveiro, which corresponds to a sex ratio of 46% for females and 54% for females. The values obtained do not differ significantly from the ratio 1:1 (χ^2 = 0.195, p>0.05) expected.

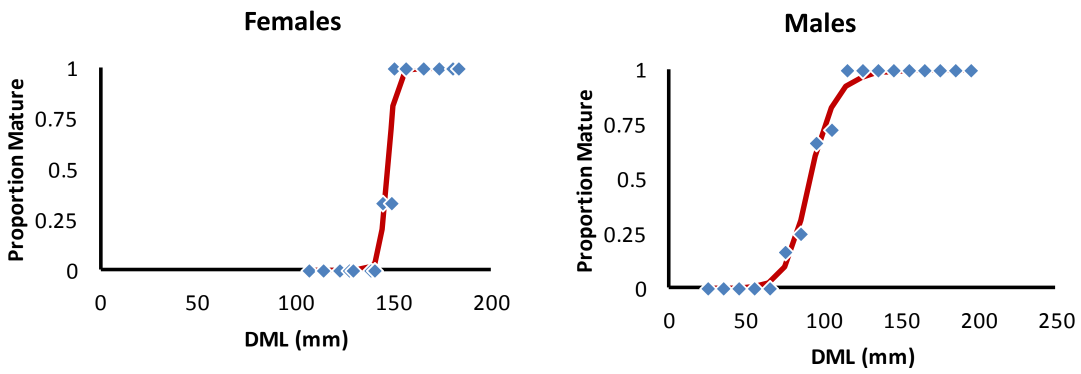

Figure II.10 – Maturity ogives for female and male *S. officinalis* according to mantle length (mm).

II.4.5. Feeding habits

A total of 208 stomachs were analysed and 32 taxa were identified, but 5 of them only occurred once. The most frequent item was *Cragon cragon* occurring in 20,19% of the stomachs, followed by *Portunidae n.i.*, *Pomatoschistus microps*, and *Mysidacea n.i.*, with a percentage of occurrences above 10%. The percentage of empty stomachs did not reach the 5%. The *Portunidae n.i.* was the preferential prey, while *Crangon crangon*, *Pomatoschistus microps* and *Carcinus maenas* were classified as secondary preys. From the identified taxa, 64% belong to Decapoda order, and 34% to the Teleostei class.

Table II-III – Numerical, gravimetric, occurrence and feed coefficient (Q).

	Numerical method	Gravimetric method	Occurrence method	Feed coefficient
Item	**C_N (%)**	**C_p (%)**	**C_f (%)**	**Q**
Detritus	3.34	2.78	6.73	9.29
Vegetable	2.63	0.03	5.29	0.08
Polychaeta n.i.	1.19	0.37	2.40	0.44
Bivalvia n.i.	1.67	0.18	2.88	0.30
Gastropoda n.i.	3.34	0.22	1.92	0.75
Mysidacea n.i.	8.35	1.69	10.10	14.15
Isopoda n.i.	2.39	0.15	2.88	0.36
Sphaeromatidae n.i.	6.44	0.58	9.62	3.75
Crustacea n.i.	4.77	1.40	9.62	6.68
Amphipoda n.i.	3.34	0.33	3.37	1.10
Natantia n.i.	4.30	3.57	8.65	15.34
Processa sp.	4.30	2.65	4.33	11.38
Palaemon sp.	0.95	0.29	1.44	0.27
Palaemon serratum	1.43	0.31	1.44	0.45
Crangon crangon	14.32	6.46	20.19	**92.46**
Atelecyclus undecimdentatus	0.24	1.61	0.48	0.38
Pilumnus hirtelus	1.19	7.81	2.40	9.32
Portunidae n.i.	11.69	17.30	15.38	**202.28**
Liocarcinus sp.	2.15	4.49	3.85	9.64
Liocarcinus marmoreus	0.95	3.73	1.92	3.56
Carcinus maenas	2.15	10.33	4.33	**22.18**
Teleostei n.i.	5.25	3.34	9.62	17.55
Gobiidae n.i.	0.72	0.99	1.44	0.71
Gobius niger	0.72	0.15	1.44	0.11
Pomatoschistus sp.	0.48	0.12	0.96	0.06
Pomatoschistus microps	6.68	6.75	10.58	**45.08**
Pomatoschistus minutus	1.67	1.87	3.37	3.13
Sparidae n.i.	0.24	0.002	0.48	0.00
Diplodus sp.	0.24	12.75	0.48	3.04
Pleuronectiformes n.i.	0.24	1.21	0.48	0.29
Syngnathidae n.i.	1.67	2.69	3.37	4.50
Syngnathus acus	0.72	2.13	1.44	1.53
Symphodus sp.	0.24	1.70	0.48	0.41

For ANOVA analysis data was **log** transformed. The result showed that the diet of cuttlefish presents significant differences between seasons and food item ($p<0.001$), but was similar within the different sites of the lagoon (Table II-IV).

Table II-IV - Factorial analysis of variance (ANOVA) using GLM to testing statistical comparisons between sites, seasons and item.

Factor	F	P
Seasons	$F_{1, 278} = 17.56$	<0.001 (S)
Sites	$F_{7, 278} = 0.61$	0.748 (NS)
Food Item	$F_{32, 278} = 4.85$	<0.001 (S)

Figure II.11 shows a very strong correlation between AFDW (g) and WWt (g), indeed AFDW (g) is about 10% of the WWt (g). Due to the strong correlation between these two parameters, for the statistical analysis we used AFDW (g) instead the WWt (g), once this measured is independent of the percentage of humidity of each type of food item.

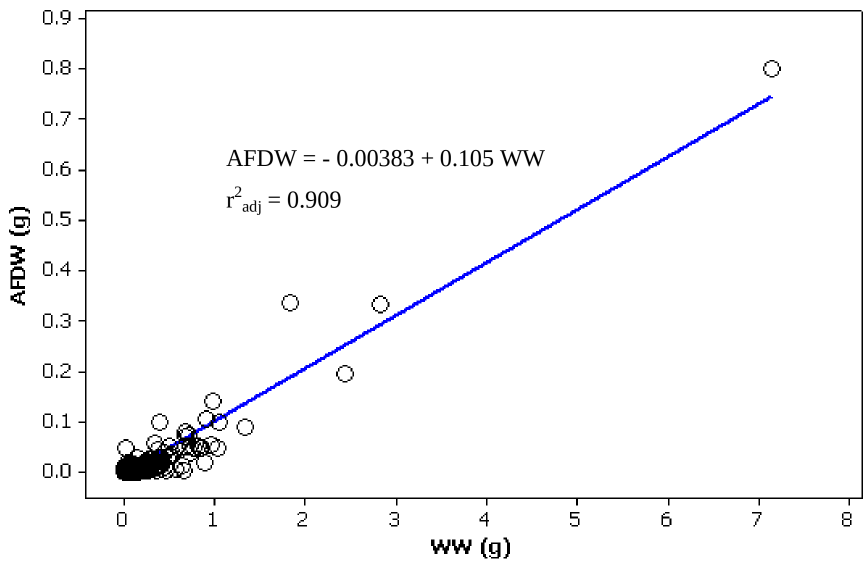

Figure II.11 - – Linear relationship between AFDW (g) and WWt (g) of guts analysed from cuttlefishes of Ria de Aveiro.

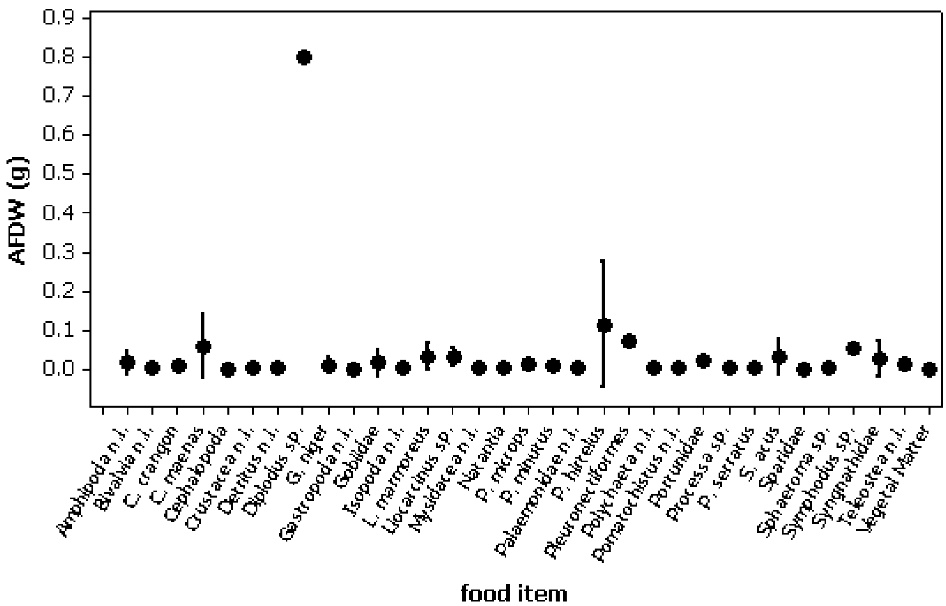

Figure II.12 – Food item distribution according to AFDW (g) measures.

Diplodus sp. was the food item that most contribute for the total weight of AFDW (g), followed by *Pilumnus hirtellus* (Phir), Pleuronectiformes, *Symphodus* sp. and *Carcinus* maenas (Figure II.12).

II.5.Discussion

The Ria de Aveiro, similar to other coastal estuarine lagoons (Leeuwen *et al.*, 1994; Elliot and Hemingway, 2002), is a system highly influenced by abiotic variations, which affect the recruitment and the survival of several species (Rebelo, 1992; Pombo and Rebelo, 2002). *S. officinalis* presented a wide dispersion in the Ria de Aveiro, where Areão (ARE), southern site of the lagoon, in the Mira channel, was the only site of the lagoon where cuttlefish was not found. Depth and salinity were the two main external factors affecting the spatial distribution of the cuttlefish in the lagoon. ARE could represent the minimum quota in terms of salinity and depths for cuttlefish presence. Other authors referred that cuttlefish tolerate values of salinity between 25 (Palmegiano and D'Apote, 1983) and 38 (Boletzky, 1983). Several authors referred that external parameters such as salinity, temperature, dissolved oxygen, turbidity, etc. affect the development of the *Sepia officinalis* life cycle (Palmegiano and D'apote, 1983; Bouchaud and Daguzan, 1990; Domingues *et al.*, 2001b). Temperature clearly plays a major role in determining the life span of *S. officinalis* (Richard, 1971; Forsythe *et al.* 1991, 1994). Indeed, the Cuttlefish abundance in Ria de Aveiro lagoon starts to increase in April, when water temperature begins to increase and reproduction is triggered. In autumn, adults seem to disappear. This behavior could result from the mass mortality after spawning or the return of adults to sea. The pattern of migration found in Ria de Aveiro is in accordance to Richard (1971), Boletzky (1983) and Martins (1990). *S. officinalis* migrates inshore in spring and summer and move offshore in autumn. During the warm seasons (spring and summer), cuttlefish migrates to coastal waters, where physical conditions are more stable than offshore and food availability is higher. In late autumn, when temperature begins to decrease, cuttlefish migrates offshore and almost disappears from the lagoon. Abundance was much higher in summer then in autumn, while spring presented the higher biomass. This abundance pattern presented in the Ria de Aveiro is in accordance with other geographical areas, like the English Channel (Dunn, 1999; Wang *et al.*, 2003; Challier *et al.*, 2005); the Estuary of Sado in Setubal

(Portugal) (Neves *et al.*, 2009) and the French coast (Morbihan Bay) (Blanc *et al.*, 1998).

The spawning season (spring and summer) found in this study was the same as the one determined for the Estuary of Sado (Portugal) (Neves *et al.*, 2009), but different to Guerra and Castro (1988) in Ria de Vigo and to Jorge and Sobral (2004) for Ria de Aveiro. In both studies mature individuals were found throughout the year. In this studied the immature individuals dominate along the year suggesting a very young population of cuttlefish. Jorge and Sobral (2004) reported two spawning seasons for Ria the Aveiro, one more representative in June and August and other in February and October, however in this studied only one was evidenced.

The stable conditions found in Ria de Aveiro during spring and summer months, and the high diversity of habitats and high prey availability represent good conditions for reproduction, especially for smaller individuals. The development of juveniles and the differences between individuals are really dependent on the environmental conditions (Challier *et al.*, 2002; Dunn, 1999; Domingues *et al.*, 2002; Koueta and Boucaud-Camou, 2003). Individuals that are born in the lagoon during summer are likely to grow faster, reaching maturity at lower age (compared to those born in the coastal zone), and becoming mature for reproduction in their first year. Individuals born in other seasons will have poorer conditions for a rapid growth and will probably reach maturity at older ages. This phenomenon is apparently related with the time interval spent at higher temperatures (Domingues *et al.*, 2001b).

Males reach sexual maturity before the females (Gauvrit *et al.*, 1997) being usually smaller than females (Pinczon du Sel and Daguzan, 1997). In the North Atlantic, cuttlefish can mature before one year old, although many individuals mature during their second year of life (Gauvrit *et al.*, 1997). Several studies reported the first length of ripe individuals for males and females (Table II-V). All the studies presented a lower first length for maturity for males than females with the exception of Jorge and Sobral (2004). The value obtained in this work is much similar with the values presented in Mediterranean (Mangold-Wirz, 1963), in Golfo de Tunes (Ezzedine-Najal, 1984) and in south Brittany (Le Goff and Daguzan, 1991), being higher than Guerra and Castro (1988), in Ria de Vigo, Önsoy and Salman (2005), in Aegean Sea and Neves *et al.* (2009), in Estuary of Sado and much lower than Dunn *et al.* (1999), in the English Channel.

Table II-V – Mean values (mm) of first length in ripe individuals for males and females, in cuttlefish from the literature.

First lenght in ripe individuals			
males (mm)	**females (mm)**	**Location**	**Reference**
78	106	Ria de Aveiro	This study
80	110	Mediterranean	Mangold-Wirz (1963)
80	100	Golfo de Tunes	Ezzedine-Najal (1984)
60	80	Ria de Vigo	Guerra and Castro (1988)
80	110	South Brittany	Le Goff and Daguzan (1991)
146	170	English channel	Dunn et al. (1999)
95	85	Ria de Aveiro	Jorge and Sobral (2004)
70	90	Aegean Sea	Önsoy and Salman (2005)
63	85	Sado Estuary	Neves et al. (2009)

Several authors referred that temperature is determinant in *Sepia officinalis* development (Palmegiano and D'apote, 1983; Bouchaud and Daguzan, 1990; Domingues *et al.*, 2001b) since it is a poikilothermic (Forsythe and Van Heukelen, 1987), so the differences found in the first maturity length of cuttlefish in those referred areas could be the result of differences in water temperature.

The length-weight relationship in this studied showed that, females are heavier than males for individuals larger than 82.4 mm. Dunn (1999) and Neves *et al.* (2009) found the same relationship, females became heavier than males at 106 mm and 108mm in Sado Estuary and English channel, respectively. The differences between males and females weight-length relationship could be due to different sexual developments, since this development has a bigger importance for females than males (Dunn, 1999). For same sex, the differences could result from other factors like quantity and quality of diet and temperature of water (Mangold-Wirz, 1963; Hatfield, 2000; Domingues *et al.*, 2001b).

Other authors reported the occurrence of two breeder generations during the spawning period, which are recognized by their mantle length (Mangold-Wirz, 1963; Le Goff and Daguzan, 1991; Gauvrit *et al.*, 1997; Dunn *et al.*, 1999; Önsoy and Salman, 2005). Considering Le Goff and Daguzan (1991), breeders in first year of life presented an average of mantle length of 134 mm for males and 148 mm for females, while breeders of second year showed approximately 220 mm ML, for both sexes. According to these values, all mature individuals presented in this study were in their first year of life.

In Ria de Aveiro, only one breeding generation was found, with an average of 98 mm DML in females and 99 mm DML in males, which is in accordance to Jorge and Sobral (2004), which referred that both males and females reach their maturity approximately with 90 mm of DML.

The present study showed that *Sepia officinalis* feeds preferentially from Portunidae (crabs) and fish, this is in accordance with other authors like Alves *et al.* (2006); Castro and Guerra, 1990 and Pinczon du Sel *et al.* (2000). Several other taxa appears as occasional preys e.g. amphipods, worms, isopods, which was also referred in other studies (Castro and Guerra, 1990; Najai and Ktari, 1979). Differences in terms of importance of prey items in feeding habits of cuttlefish, as well the presence of more species, are probably consequences of different habitats, predator size and number of analysed guts (Castro and Guerra, 1990; Pinczon du Sel *et al.*, 2000). Cannibalism was occasionally referred by Najai and Ktari (1979); Castro and Guerra, 1990), which is in accordance with this study. Predators that feed on a wide variety of preys from different habitats have a generalist diet as well as opportunistic, attacking all prey within the physical capability of the predator (Hughes, 1980). Opportunistic predation is typical of cephalopods (Boucher-Rodoni *et al.*, 1987). In this study, due to the wide range of preys found in the diet of *Sepia officinalis*, the assumptions refered above do apply to cuttlefish of the Aveiro lagoon.

II.6.Conclusions

Further studies in Ria de Aveiro and adjacent coastal waters would be needed to better understand the reproductive life cycle of *S. officinalis* on the north Portuguese coast and to understand the differences on the reproductive strategies of cuttlefish with other geographical regions of the world.

II.7.References

Alves, D.M., Cristo, M., Sendão, J., Borges, T.C. (2006) Diet of the cuttlefish *Sepia officinalis* (Cephalopoda: Sepidae) off the south coast of Portugal (eastern Algarve). *Journal of Marine Biology Association*, U.K., 86: 429-436.

Alonso-Alende, J.M. and Guerra, A. (1984). Crustáceos decápodeos y moluscos de la ria de Vigo (mimeo). Presentado alos prémios de investigación 1984, Excma. Diputación de Pontevedra, 152 p.

Barrosa, J.O. (1980). A Ria de Aveiro e a sua influência na economia da região. *Recursos Hídricos*, 1 (3):83-86.

Blanc, A., Pinczon du Sel, G., Daguzan, J. (1998). Habitat and diet of early stages of *Sepia officinalis* L. (Cephalopoda) in Morbihan Bay, France. *Journal of Molluscan Studies*, 64: 263-274.

Blanc, A., Pinczon du Sel, G., Daguzan, J. (1999). Relationships between length of prey/predator for the most important prey of the cuttlefish Sepia officinalis L. (Mollusca: Cephalopoda). Malacologia, 41: 139-145.

Boletzky, S.V. (1979). Growth and life-span of *Sepia officinalis* under artificial conditions. *Rapport Comunitaire International Mer Mèditeranee*, 10, 10 p.

Boletzky, S.V. (1983). *Sepia officinalis*. *In*: Boyle, P.R. (Ed.), Cephalopod Life Cycles, (Linnaues, 1758) in English channel. *Fishery research*, 40: 277-293.

Boletzky, S.V. and Hanlon, R. (1983). A review of the laboratory maintenance, rearing and culture of cephalopod mollusks. *Memoirs of the National Museum of the National Museum Victoria*, 44: 147-187.

Borrego, C., Cruz, M.M., Gonçalves, P. (1994). Estudo de avaliação da vulnerabilidade da capacidade de recepção das águas costeiras em Portugal. Relatório R 3.2 Meios receptores e suas características: (Be 1) – Ria de Aveiro, Pateira de Fermentelos e Barrinha de Mira. *Projecto nº 13/94. IDAD*, Aveiro.

Boucaud-Camou, E., Yim, M., Tresgot, E. (1985). Feeding and digestion of young *Sepia officinalis* L. (Mollusca:Cephalopoda) during post-hatching development. *Vie Milieu*, 35(3/4): 263-266.

Bouchand, O. and Daguzan, J. (1989). Etude de development l'oeuf de *Sepia officinalis* L. (Céphalopode, *Sepioidea*) en conditions expérimentales. *Haliotis*, 19: 189-200.

Bouchard, O. (1991). Etude de la reproduction de la seiche commune, *Sepia officinalis* L., dans le Golfe du Morbihan: Permiers résultats. *In*: Bouchard-Camou, E. (Ed.), Actes du 1 er Symposium international sur la Seiche. Caen, 1-3 juin 1989. Centre de Publications de L'Université de Caen, 153-166 pp.

Boucaud-Camou, E., Koueta, N., Boismery, J., Medhioub, A. (1991). The Sexual cycle of *Sepia officinalis* from the Bay of Seine. *In*: *La Seice* (E. Boucaud-Camou, ed.), 141-142. *Acta of the first international symposium on the cuttlefish Sepia*, Centre de Publications de l'Université de Caen.

Bouchand, O. and Daguzan, J. (1990). Etude expérimentale de l'influence de la temperature sur le déroulement du développement embryonnaire de la Seiche *Sepia officinalis* L. (Céphalopode, *Sepioidae*). *Cahiers de Biologie Marine*, 31: 131-145.

Boucher-Rodoni, R., Boucaud-Camou, E., Mangold, K. (1987). *Feeding and digestion, Cephalopod Life Cycles. Comparative Reviews*, Vol II. *In*: P. R. Boyle (ed.), Academic Press: London. Pp. 85-108.

Bowen, S.H. (1983). Quantitative description of the diet. *In* Fisheries techniques. Nielsen, L.A., Johnson, D.L., Lampton, S.S. (eds.). *American Fisheries Society*, 325-336.

Caragitsou, E. and Papaconstantinou, C. (1994). Feeding habits of piper (*Trigla lyra*) in the Saronikos Gulf (Greece). Journal of Applied Ichthyology, 10: 104-113.

Capitoli, R.R., M.L., Rufino, M.L., Vooren, C.M. (1995). Alimentação do Tubarão *Mustelus schmitti* springer na plataforma costeira do estado do Rio Grande do sul, Brasil. Atlântica, Rio Grande, 17: 109-122.

Castro, B.G. and Guerra, A. (1989). Feeding pattern of *Sepia officinalis* (Cephalopoda: Sepiodidea) in the Ria de Vigo (NW Spain), *Journal of marine biological association*, UK. 69: 545-553.

Castro, B.G. and Guerra, A. (1990). The diet of *Sepia officinalis* and *Sepia elegans* (Cephalopoda, Sepioidea) from the Ría de Vigo. *Scientia Marina*, 54(4): 375-388.

Challier, L., Royer, J., Robin, J-P. (2002). Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. *Aquatic Living Resources*, 15: 303-311.

Challier, L., Dunn, M.R., Robin, J.P. (2005) Trends in age-at-recruitment and juvenile growth of cuttlefish, *Sepia officinalis*, from the English channel. *ICES Journal of Marine Science*, 62: 1671-1682.

Choe, S. (1966). On the eggs, habits of the fry and growth of some cephalopoda. *Bulletin of Marine Science*, 16: 330-348.

Clarke, M.R. (1996). The role of cephalopods in the world's oceans: general conclusions and the future. Philosophical Transactions of the Royal Society of London, 351: 1105-1112.

Coelho, M.L. and Martins, M.C. (1991). Preliminary observations on the biology of *Sepia officinalis* in Ria Formosa, Portugal. *In*: *La Seice* (E. Boucaud-Camou, ed.), 141-142. *Acta of the first international symposium on the cuttlefish Sepia*, Centre de Publications de l'Université de Caen.

Dias, J.M. (2001). Contribution to the Study of Ria de Aveiro Hydrodynamics. PhD thesis. Physical Department, University of Aveiro, Portugal, p. 288. http://nmec.web.ua.pt/ficheiros/PDFs/jdias.pdf

DocaPesca of Aveiro. (2013). Total landings of cuttlefish in Aveiro Lagoon by tones captured and annual turnover per year, since 2002 to 2012. Statistical Department.

Domingues, P.M., Sykes, A., Andrade, J.P. (2001a). The use of artemia or mysids as food source hatchlings of cuttlefish (*Sepia officinalis* L.); effects on the growth and survival throughout the life cycle. *Aquaculture International*, 9: 319-331.

Domingues, P.M., Kingston, T., Sykes, A., Andrade, J.P. (2001b). Growth of young cuttlefish, *Sepia officinalis* (Linnaeus, 1758) at the upper end of the biological distribution temperature range. *Aquaculture Research*, 32: 923-930.

Domingues, P.M., Sykes, A., Andrade, J.P. (2002). The effects of temperature in the life cycle of two consecutive generations of the cuttlefish *Sepia officinalis* Linnaeus, 1758), cultured in the Algarve (South Portugal). *Aquaculture International*, 10: 207-220.

Dunn, M.R. (1999) Aspects of the stock dynamics and exploitation of cuttlefish, *Sepia officinalis* (Linnaeus, 1758), in the English Channel. *Fisheries Research*, 40: 277-293.

Elliot, M. and Hemingway, K.L. (Eds.) (2002). *Fishes in Estuaries*. Blackwell Science, Oxford.

Ezzedine-Najal, S. (1984). Reproduction de la seiche *Sepia officinalis* Linne, 1758 (Mollusque, Cephalopode) du Golfe de Tunis: étude morphologique et morphometrique. *Bulletin de l'Institut National Scientifique et Technique d'Oceanographie*. Pêche, Salammbô, 11:71-118.

Frost, W.E. (1977). The food of charr *Salvellinus willughbii* (Gunter) in Windemere. *Journal of Fish Biology*, 11: 531-547.

Forsythe, J.W. and Van Heukelem, W.F. (1987). Growth. *In:* Cephalopod life Cycles, vol II: Comparative Reviews, pp. 135-156. Ed. by P.R. Boyle *Academic Press*, London, 441pp.

Forsythe, J.W., Hanlon, R.T., DeRusha, R.H. (1991). Pilot large-scale culture of *Sepia officinalis* in biomedical research. *In*: Boucaud-Camou E. (ed.), *The cuttlefish*. Centre de publications de l'Université de Caen, pp. 313-323.

Forsythe, J.W., DeRusha, R.H., Hanlon, R.T. (1994). Growth, reproduction and life span of *Sepia officinalis* (Cephalopoda: Mollusca) cultured through seven consecutive generations. *Journal of Zoology*, London, 233:175-192.

Gauvrit, E., Le Goff, R., Daguzan, J. (1997). Reproductive cycle of the cuttlefish, *Sepia officinalis* (L.) in the Northern part of the Bay of Biscay. *Journal of Molluscan Studies*, 63: 19-28.

George, J.D. and Hadley, W.F. (1979). Food and habitat partitioning between rock bass (*Ambloplites rupestris*) and smallmouth bass (*Micropterus dolomieui*) young of the year. *Transactions of the American Fisheries Society*, 108: 253-261.

Gi, Geon. (1981). Etude des populations de seiche (*Sepia officinalis* L.) du Golfe de Gascogne. *Fra. Th.* 3º *Cycle oceano,* Nantes, pp. 272.

Glen, C.L. and Ward, F.J. (1968). "Wet" Weight as method for measuring stomach contents of Walleyes, *Stizostedion vitreum vitreum*. *Journal of the Fisheries Research Board of Canada*, 25(7): 1505-1507.

Guerra, A. and Castro, G.B. (1988). On the life cycle of *Sepia officinalis* (Cephalopoda, Sepioidea) in the Ria de Vigo (NW Spain). *Cahiers de Biologie Marine.*, 29: 395-405.

Guerra, A., Nixon, M., Castro, B.G. (1988). Initial stages of food ingestion by *Sepia officinalis* (Mollusca: Cephalopoda). *Journal of Zoology*, London, 214: 189-197.

Guerra, A. (1992). Mollusca, Cephalopoda. Fauna Ibérica. M.A. Ramos, Ed.). Vol.1. Museu Nacional de Ciencias Naturales. Consejo Superior de Investigaciones Cientificas, Madrid, pp. 327.

Guerra, A. (2006). Ecology of *Sepia officinalis*. *Vie et Milieu – Life & Environment*, 56(2): 97-107.

Hanley, J.S., Smolowitz, R., Bullis, R.A., Mebane, W.N., Gabr, H.R., Hanlon, R.T. (1998). Modified laboratory culture techniques for the European cuttlefish *Sepia officinalis*. *Biological Bulletin of the Marine Biology Laboratory Woods Hole*, 195: 223-225.

Hatfield, E.M.C. (2000). Do some it hot? Temperature as a possible determinant of variability in the growth of Patagonia squid, *Loligo gahi* (Cephalopoda: Loliginidae). *Fishery research*, 47: 27-40.

Herrán, R.A. (1988). Analisis de contenidos estomacales en peces. Revision bibliografica de los objectivos y la metodologia. *Informes Tecnicos. Instituto Espanol de Oceanografia*, 63, pp. 88.

Hughes, R.N. (1980). Optimal foraging theory in the marine context. *Oceanography and Marine Biology Annual Review*, 18: 423-481.

Hureau, J.C. (1970). Biologie compare de quelques poisons antarctiques (Nototheniidae). *Bulletin de l'Institut Oceanographique*, 68 (1391): 1-250.

Hynes, H.B.N. (1950). The food of fresh-water sticklebacks (*Gasterosteus aculeatus* and *Pydosteus pungitius*) with review of methods used in studies of the food of fishes. *Journal of Animal Ecology*, 19: 36-58.

Hyslop, E.J. (1980). Stomach contents analysis: a review of methods and their applications. *Journal of Fish Biology*, 17: 411-429.

Jorge, I. and Sobral, M.P. (2004). Alguns aspectos da biologia e da ecologia do choco, *Sepia officinalis* (Linnaeus, 1758), da Ria de Aveiro e do Litoral adjacente. *Relatório Técnico-Científico do IPIMAR, Série digital* (http://ipimar-iniap.ipimar.pt), nº 15, 29 p.

Koueta, N. and Boucaud-Camou, E. (1999). Food intake and growth in reared early cuttlefish *Sepia officinalis* L. (Mollusca:Cephalopoda). *Journal of Experimental Marine Biology and Ecology,* 240: 93-109.

Koueta, N., Boucaud-Camou, E. and Noel, B. (2002).Effects ofenriched natural dieta on survival and growth of juvenile cuttlefish *Sepia officinalis* L. *Aquaculture*, 203: 293-310.

Koueta, N. and Boucaud-Camou, E. (2003). Combined effects of photoperiod and feeding frequency on survival and growth of juvenile cuttlefish *Sepia officinalis* L. in experimental rearing. *Journal of Experimental Marine Biology and Ecology*, 296: 215-226.

Kromov, D.N., Lu, C.C., Guerra, A., Dong, Zh., Boletzky S.V. (1998). A synopsis of Sepiidae outside Australian waters, Smith. *Contributions to Zoology*, 586: 77-157.

Le Goeff, R. and Daguzan, J. (1991). Growth and life cycles of the cuttlefish *sepia officinalis L.* (Mollusca: Cephalopoda) in the South Britany (France). *Bulletin of Marine Science*, 49: 341-348.

Leeuwen, P.I. van, Rijnsdorp, A.D., Vingerhoed, B. (1994). Variations in abundance and distribution of demersal fish species in the coastal zone of the Southeastern North Sea between 1980 and 1993. *Demersal Fish Committee,* 10: 1-19.

Madenjian, C.P., Tyson, J.T., Knight, R.L, Kersher, M.W., Hansen, M.J. (1996). First-year growth, recruitment, and maturity of walleyes in western Lake Erie. *Transactions of the American Fisheries Society*, 125(6): 821-830.

Mangold-Wirz, K. (1963). Biologie des Céphalopodes bentiques et nectoniques de la Mer Catalane. *Vie Milieu*, suppl. 13: 1-285.

Mangold, K. (1966). *Sepia officinalis* de la mer Catalane.*Vie et Milieu,* 17:961-1012.

Mangold, K. (1983). *Octopus vulgaris*. In: Boyle P. (Ed.), Cephalopod Life Cycles, Species Accounts, vol. I. *Academic Press*, United Kingdom, 335-364.

Martins, M.C. (1990). Revisão da espécie *Sepia officinalis, 1758* e um estudo preliminar da sua biologia e pesca na região algarvia. Relatório de Estágio da Licenciatura de Biologia Marinha e Pescas, Universidade de Faro, 70 p.

Najaï, S. and Ktari, M.H. (1979). Etude du régime alimentaire de la seiche commune *Sepia officinalis* L. du Golfe de Tunis. *Bulletin de l'Institut National Scientifique et Technique d'Oceanographie*. Pêche, Salammbô, Tunisie, 6(1-4): 53-61.

Neves, A., Cabral, H., Sequeira, V., Figueiredo, I., Moura, T. and Gordo, L.S. (2009) Distribution patterns and reproduction of the cuttlefish *Sepia Officinalis* in the Sado estuary (Portugal) *Journal of the Marine Biological Association of the United Kingdom,* 89 (3): 579-584.

Nixon, M. and Mangold, K. (1998). The early life of *Sepia officinalis*, and the contrast with that of *Octopus vulgaris* (cephalopoda). *Journal of Zoology*, London 254: 401-425.

Norman, M. (2000). Cephalopods – A world Guide, Conchbooks, Germany.

Oliveira, M.R.M.D. (1997). Ecologia alimentar de *Tripterygion delaisi* Cadenat & Blache (1971) (Pisces: Tripterygiidae) dos Açores: análise faseada do tracto digestivo; variação temporal e sexual da dieta. Relatório de estágio do curso de licenciatura de Biologia Marinha e Pescas. Universidade do Algarve. Faro.

Önsoy, B. and Salman, A. (2005). Reproductive biology of the common cuttlefish *Sepia officinalis* L. (Sepiidae: Cephalopoda) in Aegean Sea. *Turkish Journal of Veterinary and animal Sciences,* 29: 613-619.

Palmegiano, G.B. and D'Apote, M.P. (1983). Combinated effects of temperatura and salinity on cuttlefish (*Sepia officinalis* L.) hatching. *Aquaculture*, 35: 259-264.

Pascual, E. (1978). Crescimiento y alimentacion de tres generaciones de *Sepia officinalis* en cultivo. *Investigacion Pesquera*, 42: 421-442.

Pastorinho, M.R., Telfer, T.C., Nogueira, A.J.A., Soares, A.M.V.M., Ranville, J.F. (2012). An evaluation of trace metal distribution, enrichment factos and risk in sediments of a coastal lagoon (Ria de Aveiro, Portugal). Environmental Earth Science, DOI: 10.1007/s12665-012-1643-xOnline First™.

Pedley, R.B. and Jones, J.W. (1978). The comparative feeding behavior of brown trout, *Salmo trutta* L. and atlantic salmon, *Salmo salar* L. in Llyn Dwythwch, wales. *Journal of Fish Biology*, 12: 239-256.

Pillay, T.V.R. (1952). A Critique of the methods of study of food of fishes. Journal of Zoology Society *of London*. India, 4(2): 185-200.

Pinczon du Sel, G. and Daguzan, J. (1992). Contribution à la connaissance du regime alimentaira de la Seiche *Sepia officinalis* L.; (Mollusque Céphalopode) dans le nrd du Golfe de Gascogne et dans le Golfe de Morbihan: Résultats préliminaires. *In*: *Les Mollusques Marins, Biologie et Aquaculture*, Actes de colloque, 14: 155-1722.

Pinczon du Sel, G. and Daguzan, J. (1997). A note on sex ratio, length and diet of a population of cuttlefish *Sepia officinalis* L. (Mollusca:Cephalopoda) sampled by three fishing methods. *Fisheries Research*, 32: 191-195.

Pinczon du Sel, G., Blanc, A., Daguzan, J. (2000). The diet of the cuttlefish *Sepia Officinalis* L. (mollusc:cephalopoda)) during its life cycle in the Northern Bay of Biscay (France). *Aquatic Sciences*, 61:167-178.

Pombo, L. and Rebelo, J.E. (2002). Spatial and temporal organisation of a coastal lagoon fish community, Ria de aveiro, Portugal. *Cybium*, 26 (3): 185-196.

Rebelo, J.E. (1992). The ichthyofauna and abiotic hydrological environment of the Ria de Aveiro, Portugal. *Estuaries*, 15 (3): 403-415.

Richard, A. (1971). Contribution à l´´etude expérimentale de la croissance et de la maturation sexuelle de *Sepia officinalis* L. (Mollusque céphalopode). PhD thesis, University of Lille, France.

Roper, C. F. E., Sweeney, C.E., Nauen, C.E. (1984). FAO Species catalogue. Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. *FAO Fisheries Synopsis*, 125(3): 277.

Sparks, T.H., Scott, W.A., Clarke, R.T. (1999). Traditional multivariate techniques: Potential for use in ecotoxicology. *Environmental Toxicology and Chemistry*, 18 (2): 128-137.

Ter Braak, C.J.F. and Smilauer, P. (2002). CANOCO reference manual and Canodraw for Windows user´s guide software for canonical ordination (version 4.5). *Biometris, Wageningen University and Research Center,* Wageningen, pp. 500.

Valente, A.C.N. (1992). Alimentação natural dos peixes (métodos de estudo). Série “Monografias” 4. Instituto de Zoologia “Dr. Augusto Nobre”, Universidade do Porto. Porto.

Valentim, J.M., Vaz, N., Silva, H., Duarte B., Caçador, I., Dias, J.M. (2013). Tagus estuary and Ria de Aveiro salt marsh dynamics and the impact of sea level rise. *Estuarine, Coastal and Shelf Science*, 1-14.

Van den Brink, P. J., Van den Brink, N.W.V., Ter Braak, C. J. F. Multivariate Analysis of Ecotoxicological data using ordination: Demonstrations of utility on the basis of various examples. *Australian Journal of ecotoxicology*, 9: 141-156.

Wang, J., Pierce, G.J., Boyle, P.R., Denis, V., Robin, J.-P, Bellido, J.M. (2003). Spatial and temporal patterns of cuttlefish (*Sepia officinalis*) abundance and environmental influences – a case study using trawl fishery data in French atlantic coastal, English Channel, and adjacent waters. *ICES Journal of Marine Science,* 60: 1149-1158.

Windell, J.T. and Bowen, S.H. (1978). Methods for study of fish diets based on analysis of stomach contents, in Methods for assessment of fish production in fresh waters. Bagenal T. (ed.), 219-226.

CHAPTER III

Accumulation of metals in common cuttlefish (*Sepia officinalis*) in a temperate lagoon

III. Accumulation of metals in common cuttlefish (*Sepia officinalis*) in a temperate lagoon

III.1. Abstract

Concentrations of essential (Fe, Zn, Cu) and non-essential (Cd and Pb) metals were determined in the tissues (mantle and digestive gland) of 53 individuals of the common cuttlefish *Sepia officinalis* (Linnaeus, 1758), captured in six different sites in the Ria de Aveiro from March to December of 2003. Over decades, these types of marine ecosystems have been subject to discharges of effluents as a result of industrial and harbour activities, as well as urban and agricultural wastes. In this study, like other European studies on cephalopods, metal concentrations in digestive gland were much higher than those found in mantle, which suggests that digestive gland is the target organ for storage of these elements, acting as a detoxification organ. Size, sex and season don´t interfere with metal concentration. The metal concentrations obtained in each analysed tissue follow the gradient: Zn>Fe>Cu>Cd>Pb. The results suggest a gradient of contamination in the direction of Laranjo. However, in terms of metal concentrations, the Ria de Aveiro can be divided in two distinct regions, the first one comprising the site LAR, and the second one including all the others. All individuals captured in LAR presented levels of Cd in mantle higher than the limit proposed for human consumption (1 µg g^{-1} wet weight). For Pb the most problematic area is TOR presenting almost all the values higher than the limit for human consumption (1 µg g^{-1} wet weight). Cadmium is positively correlated with Cu in the digestive gland of the specimens collected in Ria de Aveiro. The ratios of Cd:Zn and Cd:Cu presented significant differences in the different sites of sampling.

III.2. Introduction

Cephalopods eat a great variety of organisms, being carnivorous opportunistic predators with very high growth efficiency, and a rapid maturation that is followed by a single spawning season and, with few exceptions, an early death (Boyle, 1990). They are also consumed in large quantities by higher trophic levels, such as mammals, seabirds and fish, which support the idea that cephalopods are potentially capable of supporting significantly increased levels of exploitation (Boyle, 1990). For this reason this group of marine molluscs are considered as key species in several marine ecosystems (Amaratunga, 1983; Rodhouse, 1989; Bustamante, 2002).

Cephalopods are known for their ability to accumulate high levels of essential elements to metabolic functions as well as non-essential elements (Martin and Flegal, 1975; Miramand and Bentley, 1992; Bustamante *et al*., 1998a, b, 2000; Raimundo *et al*., 2004). Furthermore, metal levels in cephalopods, which are extensively fished and consumed by humans, are also of direct concern for public health (Bustamante *et al*., 2004). Various studies have reported metal concentrations in cephalopods from several regions such as Smith *et al*., (1984); Miramand and Bentley, (1992); Bustamante *et al*., (2000); Raimundo *et al*., (2005); Pereira *et al*., (2009). At Ria de Aveiro only one study (Pereira *et al.*, 2009) addressed this issue, but it is based in a narrow site selection and didn´t explore the spatial variability, that may be relevant due to the presence of industrial contaminated effluents.

Sediments in coastal systems, which are surrounded by urbanized and industrialized areas, may contain high quantities of metals that may become available to benthic organisms and eventually transferred to upper levels, thus affecting the marine food chain (Bryan and Langston, 1992; Lee and Luoma, 1998; Warren *et al.*, 1998), or be remobilized when sediments are dredged and disposed into water bodies (Newell *et al*., 1998). The Ria de Aveiro is a shallow productive coastal lagoon, located on the west coast of Portugal, with a complex network of inner channels into which industrial and domestic effluents have been discharged for many years (Hall, 1982). Cuttlefish, *Sepia officinalis*, is economically important target specie for Portuguese fisheries (Serrano, 1992). Taking into account the economic importance of cuttlefish (*Sepia officinalis*) and the absence of reported information about metal concentrations in closed and anthropogenic influenced ecosystems, like Ria the Aveiro, the present study proposed the assessment of metal concentrations in cuttlefish from several locations of the inner part of the Ria de Aveiro.

III.3. Materials and methods

III.3.1. Study Area

The Ria de Aveiro (40°38´N, 8°44´W) (Figure III.1) is a very important coastal system in the Portuguese west coast, with an adjacent area of about 250 km^2 (Picado *et al.*, 2010). The lagoon has a maximum width of 10 km and its length measured along the longitudinal axis is 45 km (Dias and Lopes, 2006). The average depth of the lagoon compared to the mean sea level is about 3 m, although the inlet channel can exceed 28 m deep, due to dredging operations that are regularly carried out to allow the navigation (Picado *et al.*, 2010). This coastal ecosystem is permanently connected to the sea (350 m width) and was first stabilized by man (Plecha *et al.*, 2007). The hydrodynamics of the lagoon is essentially dominated by tidal currents entering its mouth (Hall *et al.*, 1985; Dias *et al.*, 2000; Lopes and Dias, 2007) which are responsible for the mixes of freshwater and seawater. Rivers have small overall contribution in terms of water inputs, when compared with the tidal prism, but have a long-term influence in the residual transport (Lopes and Dias, 2007). The four major rivers converging to the Ria are Vouga and Antuã Rivers from the East, Caster River from the North and Boco River from the Southeast. The fresh water input in a spring tide is estimated in 1.8 x 10^6 m^3 (Moreira *et al.*, 1993; Dias *et al.*, 2001), while the tidal prism can range from 35 to 137 x 10^6 m^3 (Dias *et al.*, 2001; Lillebø *et al.*, 2011). The

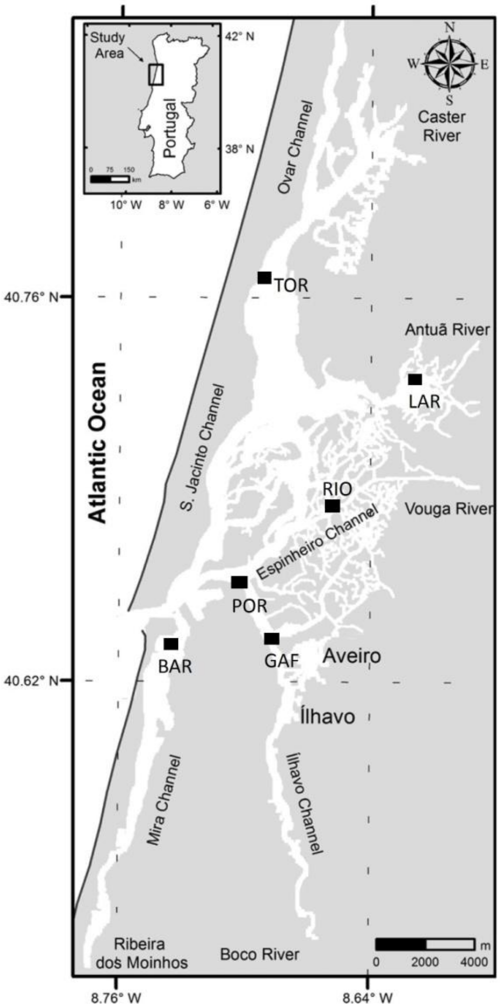

Figure III.1 - Sampling sites of *S. officinalis*: Ria de Aveiro (Adapted from Valentim *et al.*, 2013).

major freshwater input comes from the Vouga River (50m^3 s^{-1} average flow) and Antuã River (5m^3 s^{-1} average flow). The Vouga River contributes with around 2/3 of the fluvial water entering the lagoon (Moreira *et al*., 1993; Dias *et al*., 1999).

In terms of sediments quality, the lagoon receives considerable flows of raw and treated wastewater. Three main sources of pollution are apparent: organic and chemical pollution from paper-pulp factories (in rivers Vouga and Caima); chemical pollution, particularly mercury, from the industrial area of Estarreja (in Laranjo), and microbial contaminants from the urban sewage effluent and cattle raising areas (Ílhavo channel, Vouga river and Ovar channel) (Lima, 1986; Lucas *et al*., 1986; Borrego *et al.*, 1994; Pastorinho *et al*., 2012).

III.3.2. Sampling and biological data collection

A total of 53 cuttlefishes, *S. officinalis*, were caught monthly with a network trammel, from March 2003 to December 2003, at six sites (Figure III.1): near the mouth of the lagoon (BAR, POR and GAF); in the main freshwater section, highly organically enriched (RIO); in a contaminated area, result of industrial pollution (LAR), and approximately in the middle of the longest channel (TOR). The abiotic parameters such as temperature, dissolved oxygen, pH, salinity, turbidity and depth were registered on each capture. Temperature (± 0.1 ºC) and dissolved oxygen (± 0.01 mg.l^{-1}) were recorded with an oxygen meter (Consort Z621), the pH (± 0.01) with pH meter (*WTW* 330/set – 2), the salinity (± 0.1) with a refractometer (*Atago*), the turbidity (± 0.1 m) was recorded with a Secchi disc and the depth (± 0.1m) with a handmade probe. Dorsal mantle length (DML, size), total weight (W_t), sex and maturity stage were determined for each individual. All individuals were included in an allometric relationship between total weight (g) and mantle length (mm) (Figure III.2). The number of individuals (n), wet weight range (g), size range (mm), sex and maturity stage from each site is given in Table III-II. A standard 5-point scale of sexual maturity for *S. officinalis* was used (adapted from Alonso-Allende and Guerra, 1984), in which stage I is “immature”, stages II-III are ”maturing”, and stages IV-V are “mature”. Specimens with no visible gonad development were classified as “juveniles” (Challier *et al.*, 2002) and allocated in maturity stage 1. Specimens were stored in individual plastic bags and immediately frozen, in order to minimize mobilization of metals between organs (Martin and Flegal, 1975). In the laboratory, the digestive gland of each specimen was totally removed under defrost conditions, without rupture of the outer membrane, and weighted. Samples of mantle free from skin and inner membrane were also taken.

III.3.3. Analysis of metals

Tissue samples were lyophilised and homogenised. Approximately 200 mg of the dry tissue was digested with a mixture of HNO_3 (sp, 65% v/v) and H_2O_2 (sp, 30% v/v) at different temperatures according to the method described in Ferreira *et al*. (1990). All laboratory ware was cleaned with HNO_3 (20%) for 2 days and rinsed with Milli-Q water to avoid contamination. The selection of the analytical methodology and apparatus to use in metal quantification was determined by metal concentration present in the tissues. Quantification of Fe, Zn and Cu in mantle and digestive gland and Cd in digestive gland was performed by flame atomic absorption spectrometry (Perkin Elmer Analyst 100) while quantification of Cd in mantle and Pb in the mantle and digestive gland were analysed using ICP-MS. The accuracy of the analytical methods was assessed by the analysis of international certificate standards: DORM-1, DORM-2 (dogfish muscle) and TORT-1, TORT-2 (lobster hepatopancreas) (National Research Council of Canada). Blanks and standard references materials were run together with samples. The values obtained and the certificated values do not differ significantly ($p<0.05$) (Table III-I). Concentration of metal was expressed relative to both the dry weight (μg g^{-1} DWt) and the wet weight of the tissue (μg g^{-1} WWt). The dry weight values are used for statistical analysis, to eliminate any effects of varying moisture content while the wet weight is used for calculating risks associated with consumption of cuttlefish tissue (Pierce *et al.*, 2008). The values in wet weight were calculated with the percentage of humidity of each tissue. Under our analysis conditions, the detection limits (μg g^{-1}) were: 4.2 (Fe); 0.50 (Zn), 1.2 (Cu), 0.089 (for Cd in Perkin Elmer Analyst 100) and 0.0055 (for Cd in ICP-MS) and 0.0040 (Pb). Precision varied between 1 and 8%.

Table III-I - Concentration of metals in certified reference materials used to accuracy analytical quality control (mean±SD; μg g^{-1} dry weight).

Standard tissue		Fe (μg g^{-1})	Zn (μg g^{-1})	Cu (μg g^{-1})	Cd (μg g^{-1})	Pb (μg g^{-1})
DORM-1	Present study	61.7±2.8	20.4±1.7	5.95±0.48	0.092±0.009	0.30±0.12
	Certified	63.5±5.3	21.3±1.0	5.22±0.33	0.086±0.012	0.40±0.12
DORM-2	Present study	-	24.0±2.0	-	0.041±0.002	0.05±0.01
	Certified	-	25.6±2.3	-	0.043±0.008	0.07±0.01
TORT-1	Present study	186.0±12.0	-	-	25.0±0.6	9.10±0.60
	Certified	186.0±11.0	-	-	26.3±2.1	10.40±2.00
TORT-2	Present study	93.5±2.1	177.0±0.8	98±2	-	0.51±0.16
	Certified	105.0±13.0	180.0±6.0	106±10	-	0.35±0.13

III.3.4. Statistical analysis

Specific weight metal concentrations were calculated as a function of dry weight. Normality of data and homogeneity of variance were tested using the Anderson-Darling test and Levene´s test, respectively. Whenever significant deviations from normality and homoscedasticity were found, data was log-transformed prior to further statistical analysis to correct those deviations. For each metal, statistical comparisons between sites, and sex were performed using a two-way ANOVA followed by post hoc Tukey comparisons, whenever significant differences were found. Pairing site and season was also used to assess seasonality effects using a two way ANOVA. Statistical testing was carried out with MINITAB® Release 14.20. A significance level of 0.05 was used for all tests. Redundancy Analysis (RDA), a technique that falls between multiple regression and canonical correlation analysis (Sparks *et al*., 1999), was performed using the CANOCO software package for windows, version 4.5 to assess effects of environmental conditions on metal content. The significance of RDA was tested using a Monte Carlo permutation test using routines built into the software package. A complete description of the method is provided in Van den Brink *et al.* (2003).

III.4. Results

III.4.1. Relationship between DML and total weight (Wt)

Despite of the relatively low number of specimens captured, the data analysed includes a wide range of size and weight, both sexes (male (♂) and female (♀)) and individuals in maturation (stage II-III) and mature (stage IV) (Table III-II). The absence of immature individuals is associated with the selectivity of the fishing gear used, stretched mesh sizes were 80 mm. The relationship between size and weight of all cuttlefishes analysed showed a strong geometrical relationship ($r=0.95$, $n=53$, $p<0.05$), independently of maturation stage (Figure III.2), and site location (Table III-III). Furthermore, the mature stage includes a broad range of size, including individuals from 108 to 208 mm.

Table III-II - Number of individuals (n), wet weight range (g), size range (mm), sex and maturity stage of *S. officinalis* caught in the different sites of the Ria de Aveiro.

Sites	n	Weight range (g)	Size range (mm)	Sex	Stage of maturation
BAR	9	126.3-233.4	98-187	7♂ 2♀	II, III, IV
POR	25	115.3-1209.5	95-208	13♂ 12♀	II, III, IV
TOR	8	172.1-804.6	105-183	5♂ 3♀	III,IV
GAF	5	115.6-745.5	91-189	5♂	II, III, IV
LAR	3	214.4-914.9	140-185	1♂ 2♀	III,IV
RIO	3	175.1-208.5	108-113	3♂	IV

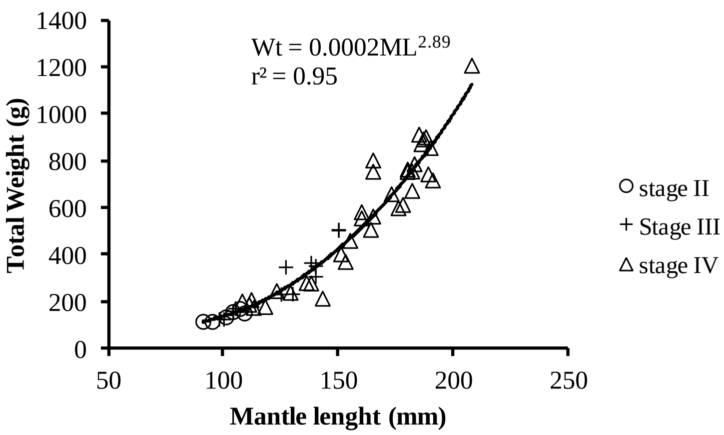

Figure III.2 - Allometric relation between total wet weight (g) and mantle length (mm) of the *S. officinalis* captured in the Ria de Aveiro, making reference to de maturity stage of each specimen (stage II and III – maturing and stage IV – mature).

III.4.2. Metal concentrations in mantle

Metal concentrations (Mean±SD; µg g^{-1} DWt) in mantle of *S. officinalis* from different sites in Ria de Aveiro are presented in Table III-III. As expected the essential elements were more abundant than the non-essential elements. Zn was the most abundant, reaching 71±4.0 µg g^{-1} DWt in GAF; followed by Fe with 28±8.0 µg g^{-1} DWt in TOR; then by Cu with 11±2.0 µg g^{-1} DWt in GAF; and the non-essential elements, Cd reach 1.24±0.12 µg g^{-1} DWt in LAR and Pb reach 1.7±2.047 µg g^{-1} DWt in TOR.

In Portugal the maximum acceptable levels of Cd and Pb for human consumption is 1.0 µg g^{-1} wet weight (WWt) [COMMISSION REGULATION (EC) Nº 629/2008 of 2 July 2008]. The average amounts of Cd in muscle of *S. officinalis,* (i.e. the tissue predominantly used for human consumption) were below this threshold, with average levels of Cd of 0.36 µg g^{-1} DWt, with the exception of specimens captured in LAR and a single individual of Rio that also presented a value higher than the limit proposed. However, the value of Cd in mantle of cuttlefish from LAR was particularly high when compared with values from other sampling sites, which presented a maximum of 0.44±0.53 µg g^{-1} DWt for RIO. The same occurs with Pb for GAF and TOR which presented values of 1.1±2.4 µg g^{-1} DWt and 1.700±2.047 µg g^{-1} DWt, respectively, while the maximum measured for other sampling sites was 0.47±1.25 µg g^{-1} DWt for BAR (Table III-III).

For mantle, there was no significant differences ($p<0.05$) between seasons. The statistical comparisons of metal concentrations in the mantle between sites and sex of the individuals (Table III-IV) showed that no significant differences ($p<0.05$) were observed between males and females for all metals. However, differences between sites were observed for: Fe in TOR where the value was significantly higher than on the other sites; Zn in LAR and GAF were significantly different from RIO, POR and BAR; and Cd levels in LAR which presented a noteworthy value that was significantly different from the other sites.

Table III-III – Metal concentrations (Mean±SD; µg g-1 DWt) and ranges in Mantle and digestive gland (DG) of *Sepia officinalis* in the sampling sites.

Sites	Fe		Zn		Cu		Cd		Pb	
	Mantle#	DG#	Mantle#	DG#	Mantle#	DG#	Mantle*	DG#	Mantle*	DG*
BAR	20±6.0	507±153	62±4	806±527	10±3	378±463	0.16±0.13	55±27	0.470±1.250	1.880±0.560
Range	15-31	348-792	54-66	58-1794	6-16	101-1679	0.02-0.32	36-93	0.004-3.800	0.710-5.330
POR	19±7.0	471±144	62±6	943±298	8±2	943±240	0.22±0.12	207±102	0.210±0.780	1.410±0.460
Range	8-35	267-852	47-74	287-1522	6-13	171-936	0.06-0.51	67-371	0.004-4.000	0.620-2.480
TOR	28±8.0	507±214	64±5	1211±285	7±2	876±365	0.19±0.14	258±78	1.700±2.047	2.800±1.880
Range	12-35	375-932	60-72	712-1576	6-9	890-1655	0.04-0.39	112-353	0.081-5.400	1.610±7.250
GAF	16±4.0	379±56	71±4	983±633	11±2	592±596	0.21±0.07	47±37	1.100±0.150	1.700±1.110
Range	12-19	313-466	65-74	355-1832	9-14	270-1654	0.12-0.26	25-112	0.021-5.300	0.710-3.550
LAR	19±4.0	891±154	70±8	1822±428	10±2	840±301	1.24±0.12	62±2	0.074±0.050	3.190±1.100
Range	15-23	716-1003	61-76	1328-2083	9-11	494-1038	0.05-1.30	60-65	0.091-0.110	2.320-4.360
RIO	11±0.3	248±84	60±2	946±419	10±2	291±227	0.44±0.53	23±3	0.021±0.011	1.630±0.560
Range	11-12	166-334	57-62	483-1299	9-13	60-515	0.06-1.00	20-26	0.010-0.030	1.050-2.180

measured with Flame Atomic Absorption Spectrometer * measured with ICP-MS

Table III-IV – Factorial analysis of variance (ANOVA) testing statistical comparisons between sites and sex for mantle and digestive gland of *S. officinalis* on the concentration of essential metals (Fe, Zn and Cu) and non-essential metals (Cd and Pb).

		Mantle			Digestive Gland		
Metals	Factors	F	Significance (p value)	Conclusions	F	Significance (p value)	Conclusions
Fe	Site	$F_{5, 46}$=4.19	0.003 (S)	RIO=GAF=POR≠TOR	$F_{5, 46}$=5.84	<0.001 (S)	RIO≠POR=TOR≠LAR
	Sex	$F_{1, 46}$=2.11	0.153 (NS)	-	$F_{1, 46}$=7.43	0.009 (S)	M≠F
Zn	Site	$F_{5, 46}$=3.56	0.008 (S)	RIO=POR=BAR≠GAF	$F_{5, 46}$=3.47	0.010 (S)	BAR=POR≠LAR
	Sex	$F_{1, 46}$=0.10	0.759 (NS)	-	$F_{1, 46}$<0.001	0.987 (NS)	-
Cu	Site	$F_{5, 46}$=2.00	0.096 (NS)	-	$F_{5, 46}$=4.16	0.003 (S)	RIO=BAR=POR≠TOR
	Sex	$F_{1, 46}$<0.001	0.961 (NS)	-	$F_{1, 46}$=0.29	0.591 (NS)	-
Cd	Site	$F_{5, 46}$=1.74	0.001 (S)	BAR=TOR=GAF=POR=RIO≠LAR	$F_{5, 46}$=2.91	<0.001 (S)	RIO=GAF=LAR=BAR≠(POR=TOR)
	Sex	$F_{1, 46}$=2.75	0.162 (NS)	-	$F_{1, 46}$=7.37	0.009 (S)	M≠F
Pb	Site	$F_{5, 46}$=2.61	0.037 (S)	POR≠TOR	$F_{5, 46}$=3.95	0.005 (S)	POR≠TOR=LAR
	Sex	$F_{1, 46}$=0.59	0.44 (NS)	-	$F_{1, 46}$=0.67	0.417 (NS)	-

III.4.3. Metal concentrations in digestive gland

Metal concentrations (Mean±SD; µg g^{-1} DWt) in digestive gland of *S. officinalis* from different sites in Ria de Aveiro are presented Table III-III. The essential metals presented higher values than the non-essential metals, similarly to the mantle. Zn was the most abundant, reaching 1822±428 µg g^{-1} DWt in LAR; followed by Cu with 943±240 µg g^{-1} DWt in POR; then by Fe with 891±154 µg g^{-1} DWt in LAR; and the non-essential elements, Cd reach 258±78 µg g^{-1} DWt in TOR and Pb reach 3.190±1.100 µg g^{-1} DWt in LAR. Along the year, the metal concentrations in digestive gland remained similar ($p<0.05$), however, between sites, metal concentrations in the digestive gland were different ($p<0.05$), and sites near the industrial area presented higher values than the farthest ones (Table III-IV).

The innermost sites presented higher metal concentrations than the sites near the mouth of the lagoon. Indeed the site LAR which is a problematic site of pollution presented the highest values of all the metals (with the exception of Cd and Cu) in the digestive gland. Torreira (TOR) was the second site showing high levels of concentration of all the metals (Table III-III). The rest of the lagoon presented a similar distribution of metals with the exception of Cd in the POR that presented very high value in comparison with others sites. For sex, only Fe and Cd showed significant ($p<0.05$) differences (Table III-IV), and for both metals, higher concentrations were found on females.

III.4.4. Metal concentration mantle vs digestive gland

Metal concentration in mantle and digestive gland in all sites, was significantly different ($p<0.05$). As expected the concentrations in the digestive gland were higher, indicating that these metals are preferentially accumulated in this organ. Depending on the sampling site, the values in digestive gland varied between one and three orders of magnitude above the mantle values (Table III-III and Figure III.3) for almost all metals studied.

III.4.5. Relationships between Cd:Zn and Cd:Cu ratios

The ratios Cd:Zn and Cd:Cu were calculated in mantle and digestive glands for all the specimens at each site (Pereira *et al.*, 2009) and ANOVA was tested to check differences between sites, sex, size and season (Table III-V). For mantle, no significant differences were found for sampling station, sex or season, although for digestive glands both ratios presented significant differences for sampling station (Table III-V). For the ratio Cd:Zn the higher value was 0.52 for POR and for the ratio Cd:Cu the higher value was 0.31 for BAR. No significant differences were found for sex and season. Moreover, ratios and tissues showed no significant differences when size of specimens was used as covariate. As observed for metal concentration in both tissues (mantle and digestive gland) also the ratios of Cd:Cu and Cd:Zn differ of one and two orders of magnitude, respectively. No correlation was observed with body weight and ratios with the exception of the ratio Cd:Cu in digestive gland that presented a positive correlation.

Table III-V - Factorial analysis of variance (ANOVA) testing statistical comparisons between sites and sex of essential (Fe, Zn and Cu) and non-essential (Cd and Pb) metals in mantle and digestive gland of *S. officinalis*.

		Mantle			**Digestive Gland**		
Ratios	Factors	F	Significance (p value)	Conclusions	F	Significance (p value)	Conclusions
Cd:Cu	Site	$F_{5,47}$=1.01	0.420 (NS)	-	$F_{5,47}$=8.53	<0.001 (S)	POR≠BAR=TOR=GAF=RIO=LAR
Cd:Zn	Site	$F_{5,47}$=1.26	0.296 (NS)	-	$F_{5,47}$=3.57	0.008 (S)	Not conclusive

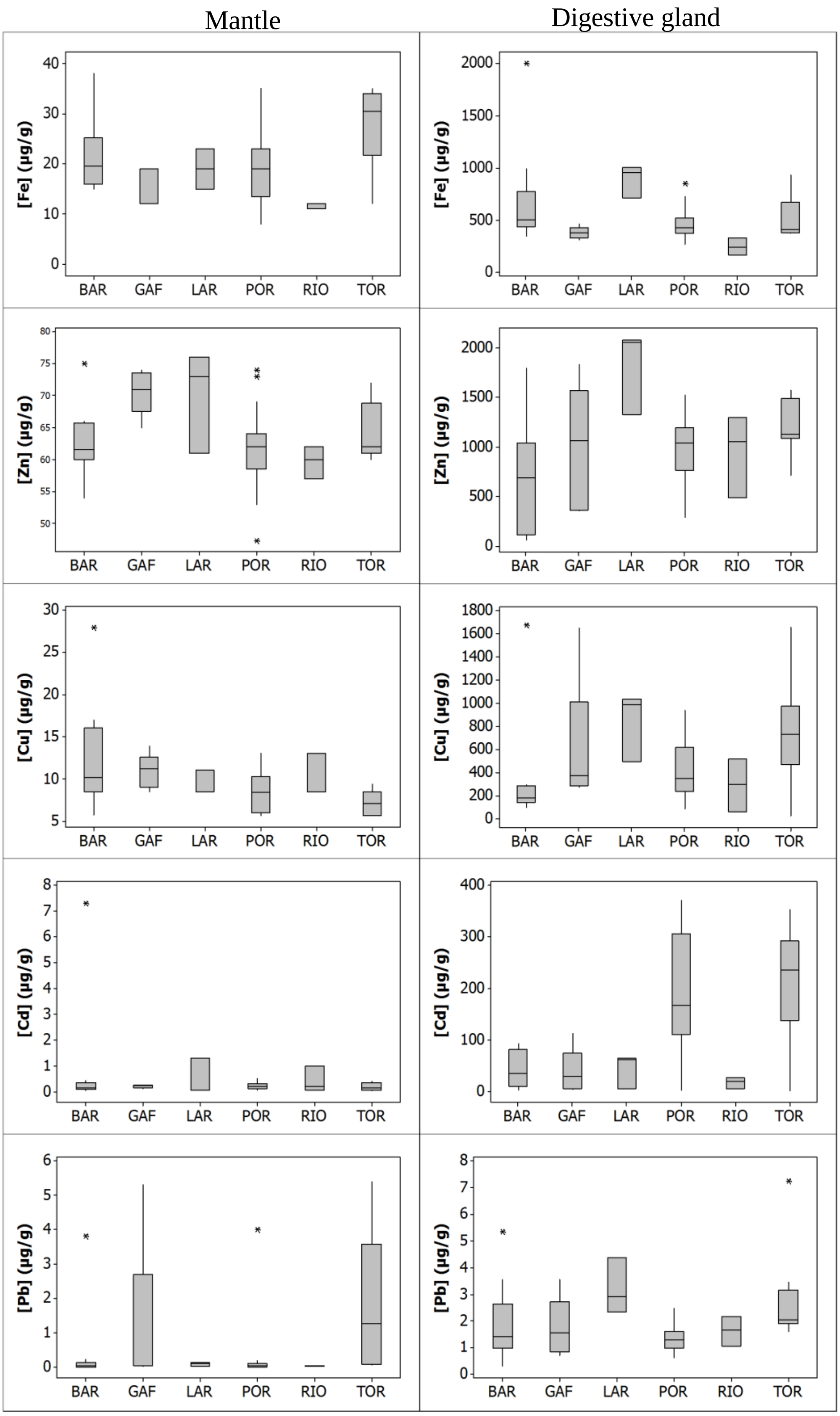

Figure III.3 - Boxplot of Fe, Cu, Zn, Cd and Pb in *S. officinalis* for mantle and digestive gland vs sites sampling (µg g^{-1} DWt).

III.4.6. Correlations between metals

In general, the correlations between metals were not significant with the exceptions of [Cu] vs [Cd] in digestive gland (p<0.05) and [Cu] vs [Pb] in digestive gland (p<0.001) (Figure III.4). Copper was positively correlated (p<0.05) to Cadmium and lead (p<0.001). Other authors have also studied these correlations, due to the quimical correlation of these metals (Pereira *et al.*, 2009).

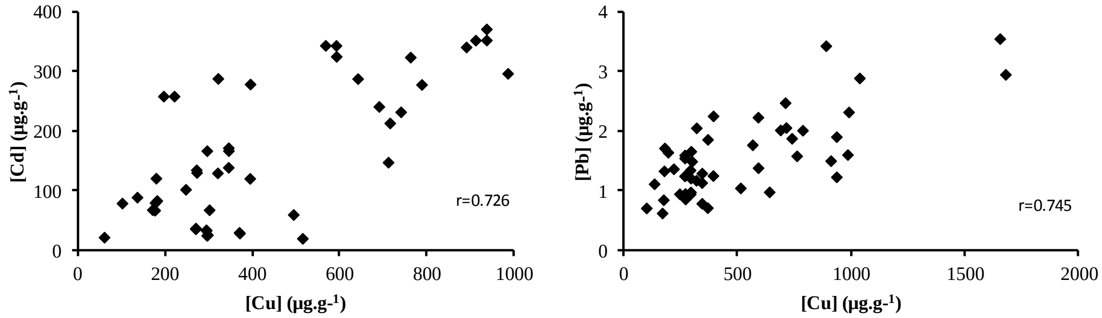

Figure III.4 – Correlations between [Cu] vs [Cd] and [Cu] vs [Pb] in the digestive glands of *S. officinalis* from Ria the Aveiro (n=53; p<0.001).

Only lead concentrations were significantly correlated in mantle and digestive gland (r=0.333; n=53; p=0.015).

III.4.7. Redundancy Analysis (RDA)

The multivariate ordination (RDA) of metal concentrations within different sites of Ria de Aveiro (Figure III.5) support the results from univariate analyses (ANOVA). However, some differences in specific element were found, because RDA considered all the elements simultaneously rather than one at the time. The first RDA axis (horizontal axis) explains 38.7 % of the total variance of metals between sites, and the second one (vertical axis) 27.5 %. Both axes explain 66.2 % of the total variability. The third axis explained only 21.1 % of the total variation, and therefore it was not considered. Monte Carlo permutation tests on RDA results showed that the first axis is statistically significant (p=0.016). Similar results were obtained when all canonical axes were considered (p=0.002). Most of the observed variability in metal concentrations in tissues of *Sepia officinalis* was associated with differences between individuals within a site (72.2 %) while 20.4 % was associated with differences between sites and 7.4 % was explained by covariates (total weight, length, and digestive gland weight). The high variability between individuals of the same sampling area hampers the establishment of patterns. Nevertheless, Laranjo is clearly separated from other sites presenting higher values of metal concentrations with the exception of Cd quantities in the digestive gland, as mentioned previously. Levels of Pb in mantle are strongly correlated with Pb concentrations in digestive gland, suggesting once more the weak affinity of the digestive gland to Pb. The opposite occurs with the accumulation of Cd in both tissues, where levels of Cd in mantle are much lower than levels of Cd in digestive gland. Torreira (TOR) is the site with higher variability, whereas POR is the area with lower variability with the exception of few values that presented extremely high values of Cd in the digestive gland. Commercial Port (POR) is strongly associated with higher depths and saturated oxygen in water. Barra presented a central distribution of metals, presenting the lowest values of metals concentrations and the factor that apparently influences more is the salinity, showing the highest values of salinity which could be explained by the proximity of the sea.

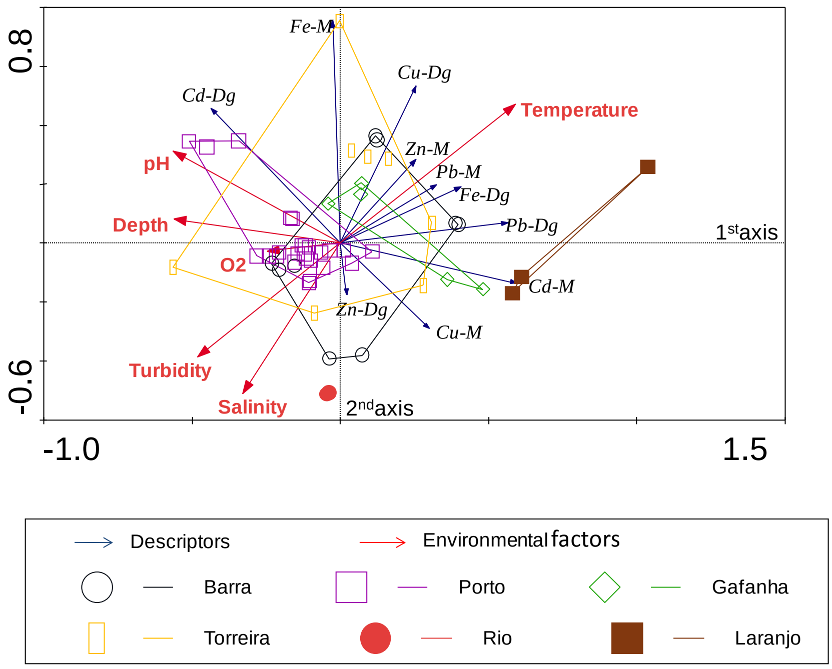

Figure III.5 - Triplot of metals concentrations in both tissues and abiotic parameters in six sites of Ria de Aveiro.

III.4.8. Relevance of metal concentration in cutlefish for human consumption

In the digestive gland all the individuals captured presented quantities of Cd and Pb higher than the limit proposed for human consumption, however for Cd the values were much higher than the limit proposed, considering the eating habits of the Portuguese people, this poses risks to human health (Figure III.6).

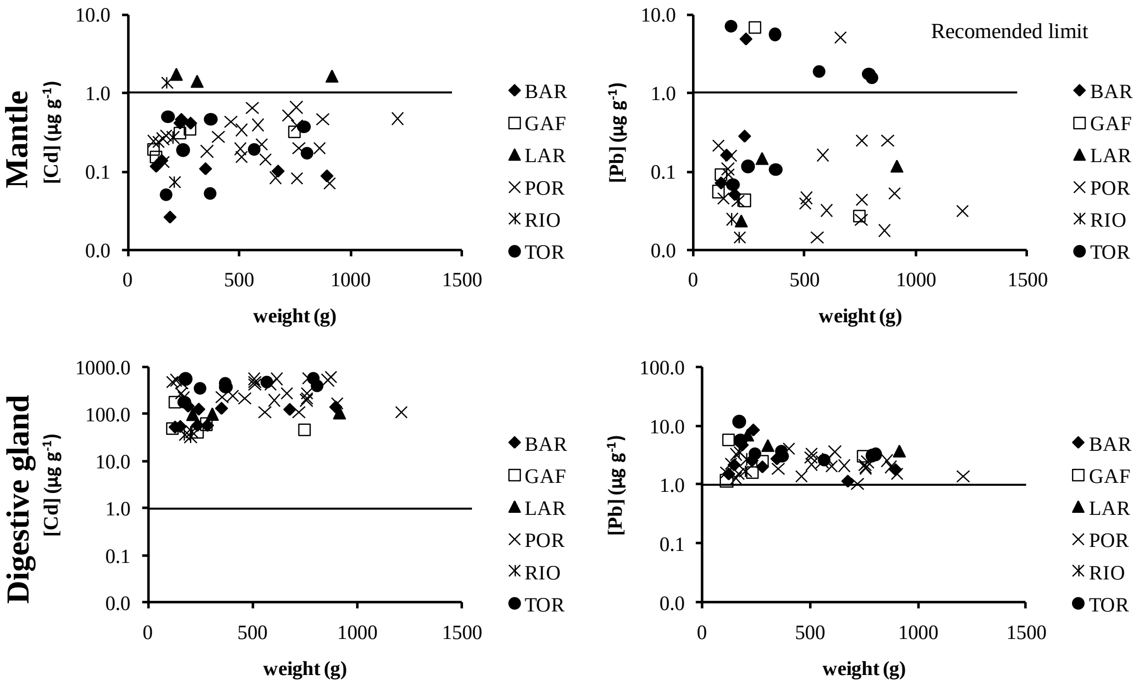

Figure III.6 – Comparison between Cd and Pb concentrations (µg g^{-1} WWt) in mantle (top graphs) and digestive gland (bottom graphs) and the limit recommended for human consumption, for the different sites of sampling.

III.5. Discussion

The strong correlation between length and weight is independent of site and season, this was also observed by others authors like Pereira (2003); Raimundo *et al.* (2005); Pereira *et al.* (2009).

The levels of Fe, Zn, Cu, Cd and Pb in the digestive gland are higher, comparing with the values in mantle. The same trend was observed in other studies with different cephalopod species (Raimundo *et al.*, 2004; Raimundo *et al.*, 2005; Pereira, 2003; Pereira *et al.*, 2009). This indicates that the accumulation of these elements in *S. officinalis* is preferentially made in the digestive gland, resulting from several mechanisms as absorption, storage and detoxification of trace elements. Various studies confirmed that the digestive gland of cephalopods concentrates high levels of Zn and Cu, and have an exceptional ability to store Cd (Miramand and Guary, 1980; Bustamante *et al*., 1998b, 2002a; Raimundo *et al.*, 2009). For TOR and GAF, the level of Pb was equal in the mantle and digestive gland (i.e. same order of magnitude) this may be caused by a higher availability of lead in these sites. This fact could be related with the location of both sites in Ria de Aveiro, GAF is situated in the vicinity of a deep-sea fishing port and dry-docks and also connected with the main channel coming from Aveiro city carrying domestic discharges, and TOR, in an intermediate region of the longest channel (S. Jacinto-Ovar channel), containing a higher number of recreational boats. However, until such assumptions are confirmed by levels of Pb in the water, they remain highly speculative.

In general, metal concentrations obtained in this study for mantle and digestive gland of *S. officinalis* were higher when compared with values reported for same species and other cephalopods of various studies (Table III-VI). Comparisons with specimens from different ecosystems must be carefully made, as identical species may exhibit different feeding habits, growth rates and habitats, which may interfere with metal accumulation (Bustamante *et al.*, 1998b; Raimundo *et al.*, 2004; Pierce *et al.*, 2008). However, the values obtained were comparable to previous data obtained along the Portuguese coast (Pereira *et al.*, 2009; Seixas *et al.*, 2005; Raimundo *et al.*, 2005, 2008). Indeed, when compared with levels of *S. officinalis* of the same area (Pereira *et al.*, 2009) the values obtained are higher or very similar, with the exception of Pb whose value was lower. For different geographic regions, the only exception was the slightly lower values of Fe, Zn and Cu obtained when compared to specimens captured in Mediterranean Sea and English Channel (Miramand and Guary, 1980; Miramand and Bentley, 1992) (Table III-VI).

The most striking aspect was the extremely high value of Cd in mantle of specimens captured in Laranjo (LAR) and the relatively low value of this element in digestive gland (when compared with values of Cd obtained for POR and TOR in the digestive gland). The increase of Cd in mantle, without a corresponding increase in the digestive gland, organ where this element is stored (Bustamante, 1998; Bustamante *et al.*, 2002a; Bustamante *et al*., 2002b; Raimundo *et al.*, 2009) suggests a large transference of Cd directly from water. This fact probably indicates an acute exposition and a recent uptake from water.

For Cd the values obtained in all sites of Ria de Aveiro are higher than the values measured in previous studies (see Table III-VI). This pattern of high levels of Cd in digestive glands of specimens captured in Ria de Aveiro appears to be a sign of higher Cd exposure. In reality, studies of distribution and accumulation of metals in sediments of Ria de Aveiro presented high Cd concentrations in the lagoon (Monterroso *et al.*, 2003a). Due to the species mobility and the different availability of preys along the lagoon, metal concentrations in tissues cannot be exclusively viewed as direct reflex of environmental conditions. However, there is no doubt that the contamination of Cd in Ria de Aveiro is essentially a result of the industrial complex of Estarreja, near the bay of Laranjo and the contamination of others locals of the lagoon are made by transport of contaminated sediments and water to the lagoon by tidal currents of Ria the Aveiro (Pereira *et al.,* 1998; Monterroso *et al*., 2003a) and by plankton exportation (Monterroso *et al*., 2003b). Bustamante *et al.* (2000) suggested that high Cd levels in digestive glands of species captured in contaminated areas are related to the surrounding environment. Commercial Port presented a really high value of Cd when compared with other sites, which could be related with the activities of the Port and also with the fact that this area forms a sort of bay which is influenced by tides that lead the contaminants into the bay and remain there accumulated. However, it´s also an area of refuge were fisheries are not allowed and probably a site where cuttlefish from other sites seek refuge. This idea might be supported by the increase number of specimens captured in this site in comparison with other sites (Table III-II).

Metal concentrations increase as the distance from the sea increase. This metal distribution pattern in digestive gland appears to reflect the location of anthropogenic sources of metals discharged in the northern region of Ria de Aveiro (Monterroso, *et al*., 2003a). Laranjo (LAR) and Torreira (TOR) are, probably, the most contaminated areas, presenting the highest values of essential (Fe, Zn, Cu) and non-essential (Cd and Pb) metals for digestive gland (Figure III.3). The higher values presented in Torreira (TOR) suggests that this local suffers the influence of Largo da Coroa, considered the second most contaminated area after Laranjo bay in Ria de Aveiro (Monterroso *et al.*, 2003a). Once more, metal concentrations in cephalopods could also vary according to the location where individuals were captured (Bustamante *et al.,* 1998b; Seixas *et al.*, 2005; Bustamante *et al.*, 2008; Pierce *et al*., 2008).

For metal-metal relationship in digestive gland and mantle, only Pb presented a low correlation between the two tissues, this could be related with the fact that food is the main source of metal contamination in cephlopods (Bustamante, 1998; Bustamante *et al*., 2002b).

Only the ratio Cd:Cu in digestive gland presented a positive correlation with wet body weight, meaning that Cd was progressively sequestrated in digestive gland with age. This pattern was also observed for *S. officinalis* in Pereira *et al.* (2009) and for *O. vulgaris* in Raimundo *et al.* (2005).

Miramand and Bentley (1992) and Bustamante (1998) showed that due to the similarities in chemical behaviour, Cd (non-essential metal), when in high concentrations in digestive gland, can compete with Zn and Cu (essential metals) for the same ligands, specific metalloproteins presented in digestive gland of cephalopods. For this reason it would be expected significant correlations between Cd-Cu and Cd-Zn, like in Pereira *et al.* (2009), however in this study it was only found significant correlations ($P<0.01$) for Cd and Cu in digestive gland. In Aveiro lagoon the specimens captured in POR (Commercial Port) presented high levels of Cd and the highest value of ratio Cd:Cu in the digestive gland, significantly different ($P<0.05$) than others (see Table III-V). Since essential elements tend to be maintained at a fairly constant concentration in tissues, Cd:Cu ratios increase in cephalopods contaminated by Cd (Bustamante *et al.*, 1998a; Raimundo *et al.*, 2005; Pereira *et al.*,2009).

In Mediterranean countries, the whole body of small cuttlefish is usually consumed. For bigger specimens, the digestive gland is often removed, but due to the high levels of Cd in the digestive gland, contamination of Cd in the mantle is frequent. This contributes significantly to Cd intake, since the mass of digestive gland is approximately 10% of the mantle, and the Cd content of other organs is negligible due to their small masses and low Cd concentrations (Miramand and Bentley, 1992), one may estimate that 95% of total body burden of Cd is stored in the digestive gland. Thus, frequent consumption of these small cuttlefish by humans might pose serious health risks.

Table III-VI – Reported metal concentrations (mean±SD µg g^{-1} or range) in different tissues of different cephalopod species.

Species	Site	Tissue	Fe	Zn	Cu	Cd	Pb	Origin	Reference
Sepiidae									
Sepia officinalis	Ria de Aveiro	Mantle	19.8±7.5	62.9±5.5	8.7±2.6	0.22±0.17	0.58±1.4	Portuguese Coast	This study
S. officinalis	Ria de Aveiro	Dig. Gland	461±159	966±394	503±386	162±114	1.74±1.15	Portuguese Coast	This study
S. officinalis	Laranjo Bay	Mantle	18.8±3.7	70.1±7.8	10.4±1.6	1.24±0.12	0.074±0.049	Ria de Aveiro, Portugal	This study
S. officinalis	Laranjo Bay	Dig.Gland	891±154	1822±428	840±301	61.7±2.4	3.19±1.06	Ria de Aveiro, Portugal	This study
S. officinalis	English Channel	Mantle	14±5	62±1	9±1	0.08±0.02	0.17±0.07	French Coast	Miramand and Bentley, 1992
S. officinalis	English Channel	Dig.Gland	244±28	571±47	315±3	12.7±0.4	1.14±0.06	French Coast	Miramand and Bentley, 1992
S. officinalis	Bay of the Seine	Dig. Gland	390±10	1400±500	600±10	25±5	2.2±0.5	River Seine	Miramand *et al.*, 2006
S.officinalis	North Coast of Portugal	Mantle	5.4-40	62-101	3.3-14	0.027-0.81	-	Portuguese Coast	Raimundo *et al.*, 2005
S.officinalis	North Coast of Portugal	Dig. Gland	156-470	220-2698	68-1761	52-557	-	Portuguese Coast	Raimundo *et al.*, 2005
S.officinalis	South Coast of Portugal	Mantle	5.9-17	62-113	4.7-19	0.010-0.065	-	Portuguese Coast	Raimundo *et al.*, 2005
S.officinalis	South Coast of Portugal	Dig. Gland	272-766	640-5678	900-5054	10-41	-	Portuguese Coast	Raimundo *et al.*, 2005
S.officinalis	Coast of Portugal	Dig. Gland	460	1435	1289	108	8.2	Portuguese Coast	Pereira *et al.*, 2009
S.officinalis	Ria de Aveiro	Dig.Gland	-	-	600	90	-	Portuguese Coast	Pereira *et al.*, 2009
S.officinalis	Ria Formosa	Dig.Gland	-	-	1000	15	-	Portuguese Coast	Pereira *et al.*, 2009
Nautilidae									
Nautilus macromphalus	Barrier reef of Nouméa	Dig. Gland	554±238	672±208	106±46	45±13	-	New Caledonia waters	Bustamante et al.,2000
Architeuthidae									
Architeuthis dux	Bay of Biscay	Dig. Gland	497±779	103±51	108±83	60.8±46.2	0.41±0.33	Atlantic Spanish Coast	Bustamante *et al.*, 2008
A. Dux	Mediterranean	Dig. Gland	158	219	1218	90.7	0.85	Mediterranean Sea	Bustamante *et al.*, 2008
Octopodidae									
Eledone cirrhosa	English Channel	Mantle	25±10	105±4	17±1	0.24±0.01	0.11±0.05	French Coast	Miramand and Bentley, 1992
Eledone cirrhosa	English Channel	Dig. Gland	267±6	646±12	456±16	23.9±1.3	1.16±0.08	French Coast	Miramand and Bentley, 1992
Octopus vulgaris	Mediterranean	Mantle	30±10	50±10	50±4	0.04±0.01	-	Mediterranean Sea	Miramand and Guary, 1980
O. Vulgaris	Mediterranean	Dig. Gland	700±130	1450±400	2500±700	50±10	-	Mediterranean Sea	Miramand and Guary, 1980
O. Vulgaris	Viana do Castelo	Arm	49±41	142±92	81±68	19±24	2.9±0.2	Portuguese Coast	Seixas *et al.*, 2005
O. Vulgaris	Cascais	Arm	41±83	74±12	14±5.5	0.4±0.0	4.0±1.0	Portuguese Coast	Seixas *et al.*, 2005
O. Vulgaris	Matosinhos and Olhão	Dig. gland	-	410-2873	639-1597	10-252	1.5-7.2	Portuguese Coast	Raimundo *et al.*, 2008

III.6. Conclusions

The digestive gland of *S. officinalis* has an extraordinary capacity to storage elements like metals, especially Zn, Cu and Cd. It seems that this organ also has the ability to redistribute these elements into the mantle of cuttlefish. For these reasons the digestive gland is considered a biomarker of potential contamination by metals in cuttlefish.

S. officinalis shows a predisposition to accumulate Cd and was considered, like other cephalopods, by Bustamante *et al.* (1998b) as a vector for transfer of Cd to top marine predators. Cuttlefishes captured in Ria de Aveiro presented, in digestive gland and in some mantles, high levels of Cd, which seems to be a response to Cd contamination in marine ecosystems. Results also suggest that there can be higher accumulation of Cd on the mantle, rather than on the digestive gland, in environmental ecosystems with high levels of Cd. Owing to this contamination level of Cd in organisms near potential sources of contamination more studies with high number of individuals should be developed to ashore the good quality of the captured cuttlefish. Until now it seems that the mechanisms of sequestration and detoxification occurring in the digestive gland are effective to ashore the normal development of cuttlefish.

Due to the local economic importance of *S. officinalis* for the region of Aveiro, and for being a target specie for this type of studies, further studies should be developed in strategic sites of the lagoon (Laranjo bay, Largo da Coroa and Commercial Port of Aveiro), may be further supplemented by studies of monitoring metal concentrations with emphasis on the function of the digestive gland as a storage organ of toxic compounds and principal producer of metalloproteins to the sequestration of these elements. Beyond that, trace elements are generally considered for their potential toxicity in ecotoxicological studies and biomonitoring surveys, and there is an increase interest in their use to provide information on life history and trophic ecology of cephalopods.

III.7. References

Alonso-Alende, J.M. and Guerra, A. (1984). Crustáceos decápodeos y moluscos de la ria de Vigo (mimeo). Presentado alos prémios de investigación 1984, Excma. Diputación de Pontevedra, 152 p.

Amaratunga, T. (1983). The role of cephalopods in the marine ecosystem. *In* I.F. Caddy, *Advances in assessment of world cephalopod resources*. *FAO Fisheries Technical Paper*, 231 (pp.379-415).

Barrosa, J.O. (1980). A Ria de Aveiro e a sua influência na economia da região. *Recursos. Hídricos.*, 1 (3): 83-96.

Borrego, C., Cruz, M.M., Gonçalves, P. (1994). Estudo de avaliação da vulnerabilidade da capacidade de recepção das águas costeiras em Protugal. Relatório R 3.2. Meios receptores e suas características: (Be 1) – Ria de Aveiro, Pateira de Fermentelos e Barrinha de Mira. Projecto nº 13/94. IDAD, Aveiro.

Boyle, P.R. (1990). Cephalopod biology in the fisheries context. *Fisheries Research*, 8 (4): 303-321.

Bryan, G.W., Langston, W.J. (1992). Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution*, 76: 89-131.

Bustamante, P. (1998). Etude des processus de bioaccumulation et de détoxification d´éléments traces (métaus lourds et terres rares) chez les mollusques cèphalopodes et bivalves pectinidés. Implication de leur biodisponibilité pour le transfert vers les prédateurs. Thesis, University of La Rochelle: pp.290.

Bustamante, P., Cherel, Y., Caurant, F., Miramand, P. (1998a) Cadmium, copper and Zinc in octopuses from Kerguelen Islands, Southern Indian Ocean. *Polar Biology*, 19: 264-271.

Bustamante, P., Caurant, F., Fowler, *S.*, Miramand, P. (1998b) Cephalopods as a vector for the transfer of Cd, to top marine predators in the North-East Atlantic Ocean. *Science of the Total Environment*, 220: 71-80.

Bustamante, P., Grigioni, *S.*, Boucher-Rodoni, R., Caurant, F., Miramand, P. (2000). Bioaccumulation of 12 trace elements in the tissues of Nautilus *Nautilus macromphalus* from New Caledonia. *Marine Pollution Bulletin*, 40: 688-696.

Bustamante, P., Cosson, R.P., Gallien, I., Caurant, F., Miramand, P. (2002a). Cadmium detoxification processes in the digestive gland of cephalopods in relation to accumulated Cd concentrations. *Marine Environmental Research*, 53: 227-241.

Bustamante, P., Teyssié, J-L., Fowler, S.W., Cotret, O., Danis, B., Miramand, P., *et al.* (2002b). Biokinetics of zinc and cadmium accumulation and depuration at different stages in the life cycle of the cuttlefish *Sepia officinalis*. *Marine Ecology Progress Series*, 231: 167-77.

Bustamante, P., Teyssié, J.-L., Danis, B., Fowler, *S.*W., Miramand, P., Cotret, O., Warnau, M. (2004). Uptake, transfer and distribution of silve rand cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. *Marine Ecology Progress Series*, 269: 185-195.

Bustamante, P, González, A.F., Rocha, F., Miramand, P., Guerra, A. (2008). Metal and metalloid concentrations in the giant squid *Architeuthis dux* from Iberian Waters. *Marine Environmental Research*, 66: 278-287.

Challier L., Royer, J., Robin, J-P. (2002). Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. *Aquatic Living Resources*, 15: 303-311.

COMMISSION REGULATION (EC) Nº 629/2008 of 2 July 2008 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, L 173/6.

Dias, J.M., Lopes, J.F., Dekeyser, I. (1999). Hydrological characterization of Ria de Aveiro, Portugal, in early summer. *Oceanologica Acta*, 22 (5): 473–485.

Dias, J., Lopes, J., Dekeyser, I. (2000). Tidal Propagation in Ria de Aveiro Lagoon, Portugal. *Physics and Chemistry of the Earth*, 4(25): 369-374.

Dias, J., Lopes, J., Dekeyser, I. (2001). Lagrangian transport of particles in Ria de Aveiro lagoon. Portugal. *Physics and Chemistry of the Earth* Part B *Hydrology, Oceans and Atmosphere* 26: 721-727.

Dias, J.M. and Lopes, J.F. (2006). Implementation and Assessment of Hydrodynamic, Salt and Heat Transport Models: The case of the Ria de Aveiro Laggon (Portugal). *Environmental Modelling & Software*, 21: 1-15.

Ferreira, A., Cortesão, C., Castro, O., Vale, C. (1990). Accumulation of metals and organochlorines in tissues of oyster *Crassostrea angukata* from the Sado estuary. *Science of Total Environment*, 97-98: 627-639.

Hall, A. (1982). Water quality problems in Ria de Aveiro. In: Actual Problems of Oceanography in Portugal, Junta Nacional de Investigação científica e Tecnológica and Nato Marine Sciences Panel (ED.), Lisboa, pp.159-169.

Hall, A., Lucas, M.F., Caldeira, M.T., Duarte, A.C. (1985). Distribuição de Mercúrio nos Sedimentos da Ria de Aveiro (Distribution of Mercury in the sediments of Ria de Aveiro, Portugal). Actas das jornadas da Ria de Aveiro. Poluição da Ria de Aveiro, I. Câmara de Aveiro, pp.103-25.

Lee, B.G. and Luoma, *S*.N. (1998). Influence of microalgal biomass on absorption efficiency of Cd, Cr, and Zn by two bivalves from San Francisco Bay. *Limnology and Oceanography,* 43 (7): 1455-1466.

Lillebø, A. I., Queiroga, H., Dias, J. M., Alves, F., Cleary, D. F. R. (2011) Ria de Aveiro: Uma Visão dos Processos Ambientais, Ecológicos e Socioeconómicos. *Actas das Jornadas da Ria de Aveiro*, Universidade de Aveiro, CESAM, pp.334-339.

Lima, C. (1986). Impacto da poluição por mercúrio nos organismos aquáticos da Ria de Aveiro. *Relatório do Instituto Nacional de Investigação das Pescas,* 66: 1-28.

Lopes, F.J., Dias, J.M. (2007). Residual circulation and sediment distribution in the Ria de Aveiro lagoon, Portugal. *Journal of Marine Systems,* 68: 507-528.

Lucas, M.F., Caldeira, M.T., Hall, A., Duarte, A.C., Lima, C. (1986). Distribution of mercury in the sediments and fishes of the lagoon of Aveiro, Portugal. *Water Science and Technology*, 18: 141-148.

Martin, J. and Flegal, A. (1975). High copper concentrations in squid levers in association with elevated levels of silver, Cd, and Zn. *Marine Biology*, 30: 51-55.

Miramand, P. and Guary, J.C. (1980). High concentrations of some heavy metals in tissuesof the Mediterranean octopus. *Bulletin of Environmental Contamination and Toxicology,* 24: 783-788.

Miramand, P. and Bentley, D. (1992). Concentration and distribution of heavy metals in tissues of two cephalopods, *Eledone cirrhosa* and *Sepia officinalis,* from the French coast of the English Channel. *Marine Biology*, 114: 407-414.

Miramand, P., Bustamante, P., Bentley, D., Kouéta, D. (2006). Variation of heavy metal concentrations (Ag, Cd, Co, Cu, Fe, Pb, V, Zn) during the life cycle of the common cuttlefish *Sepia officinalis*. *Science of the Total Environment,* 361: 132-143.

Monterroso, P., Pato, P., Pereira, E., Vale, C., Duarte, A.C. (2003a). Distribution and accumulation of metals (Cu, Cd, Zn and Pb) in sediments of a lagoon on the northwestern coast of Portugal. *Marine Pollution Bulletin,* 46: 1200-1211.

Monterroso, P., Abreu, *S*.N., Pereira, E., Vale, C., Duarte, A.C. (2003b). Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro). *Acta Oecologica,* 24: S351-S357.

Moreira, M.H., Queiroga, H., Machado, M.M., Cunha, M.R. (1993). Environmental gradients in a southern estuarine system: Ria de Aveiro, Portugal, implication for a soft bottom macrofauna colonization. *Netherlands Journal of Aquatic Ecology*, 27(2–4): 465–482.

Newell, R.C., Seiderer, L.J., Hitchcock, D.R. (1998). The impact of dredging works in coastal waters: a review of sensitivity to disturbance and subsequent recovery of biological resourses on the sea bed. *Oceanography and Marine Biology*, 36: 127-178.

Oliveira, M., Maria, V.L., Ahmad, I., Serafim, A., Bebianno, M.J., Pacheco, M., Santos, M.A. (2009). Contamination assessment of a coastal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* – An integrated biomarker approach. *Environmental Pollution,* 157: 959-967.

Pastorinho, M.R., Telfer, T.C., Nogueira, A.J.A., Soares, A.M.V.M., Ranville, J.F. (2012). An evaluation of trace metal distribution, enrichment factos and risk in sediments of a coastal lagoon (Ria de Aveiro, Portugal). *Environmental Earth Science,* DOI: 10.1007/s12665-012-1643-xOnline First™.

Pereira, M.E., Duarte, A.C., Millward, G., Vale, C., Abreu, *S*.N. (1998). Tidal export of particulate mercury from the most contaminated area of Aveiro´s Lagoon, Portugal. *Science of the Total Environment*, 213: 157-163.

Pereira, P. (2003). Repartição de metais e organoclorados em *Sepia officinalis* de dois sistemas lagunares: Ria de Aveiro e Ria Formosa. Tese de Mestrado, Universidade de Aveiro.

Pereira, P., Raimundo, J., Vale, C., Kadar, E. (2009). Metal concentrations in digestive gland and mantle of *Sepia officinalis* from two coastal lagoons of Portugal. *Science of the total Environment*, 407: 1080-1088.

Picado, A., Dias, J.M., Fortunato, A. (2010). Tidal changes in estuarine systems induced by local geomorphologic modifications. Continental Shelf Research, 30 (17), 1854-1864.

Pierce, G.J., Stowasser, A., Hastie, L.C., Bustamante, P. (2008). Geographical, seasonal and ontogenetic variation in cadmium and mercury concentrations in squid (Cephalopoda: Teuthoidea) from UK waters. *Ecotoxicology and Environmental Safety*, 70: 422-432.

Plecha, S., Rodrigues, S., Silva, P., Dias, J.M., Oliveira, A., Fortunato, A.B. (2007). Trends of bathymetric variations at a tidal inlet. *In*: Dohmen-Janssen, M., Hulscher, S. (Eds.), Proceedings of the Fifth IAHR Symposium on River, Coastal and Estuarine Morphodynamics, Enschede, The Netherlands, pp. 19–23, ISBN: 0415453631.

Pombo, L. (2005). Diversity, population dynamics and fish production capacity in a coastal lagoon – the Ria de Aveiro. Ph.D. Thesis, University of Aveiro, pp. 272.

Raimundo, J., Caetano, M., Vale, C. (2004) Geographical variation and partition of metals in tissues of *Octopus vulgaris* along the Portuguese coast. *Science of the Total Environment*, 325:71-81.

Raimundo, J., Pereira, P., Vale, C., Caetano, M. (2005). Fe, Zn, Cu and Cd concentrations in the digestive gland and muscle tissues of *Octopus vulgaris* and *Sepia officinalis* from two areas in Portugal. *Ciencias Marinas*, 31(1B): 243-251.

Raimundo, J., Vale, C., Duarte, R., Moura, I. (2008). Sub-cellular partitioning of Zn, Cu, Cd and Pb in the digestive gland of native *Octopus vulgaris* exposed to different metal concentrations (Portugal). *Science of the Total Environment*, 390: 410-416.

Raimundo, J., Pereira, P., Vale, C., Kadar, E. (2009). The Case of Exceptional Capacity of Cephalopods to Accumulate CD in the Digestive Glands. *In*: Cadmium in the Environment. *Nova Science Publishers, Inc.* ISBN: 978-1-60741-934-1.

Rodhouse, P.G. (1989). Antarctic cephalopods- a living marine resource? *Ambio*, 18(1): 56-59.

Seixas, *S.*, Bustamante, P., Pierce, G.J. (2005). Interannual patterns of variation in concentrations of trace elements in arms of *Octopus vulgaris*. *Chemosphere*, 59: 1113-1124.

Serrano, M. (1992). Contribuição para o conhecimento da biologia e pescaria do choco, *Sepia officinalis* (Linnaeus, 1758) no estuário do Sado e zona costeira adjacente. Relatório Técnico-Científico, INIP, Lisboa.

Smith, J.D., Plues, L., Heyraud, M., Cherry, R.D. (1984). Concentrations of the elements Ag, Al, Ca, Cd, Cu, Fe, Mg, Mn, Pb and Zn, and the radionuclides ^{210}Pb and ^{210}Po in the digestive gland of the squid *Notodarus gouldi*. *Marine Environmental Research.*, 13: 55-68.

Sparks, T.H., Scott, W.A., Clarke, R.T. (1999). Traditional multivariate techniques: Potential for use in ecotoxicology. *Environmental Toxicology and Chemistry*, 18 (2): 128-137.

Van den Brink, P.J., Van den Brink, N.W., Ter Braak, J.F. (2003). Multivariate Analysis of Ecotoxicological data using ordination: demonstrations of utility on the basis of various examples. *Australasian Journal of Ecotoxicology*, 9: 141-156.

Valentim, J.M., Vaz, N., Silva, H., Duarte B., Caçador, I., Dias, J.M. (2013). Tagus estuary and Ria de Aveiro salt marsh dynamics and the impact of sea level rise. Estuarine, Coastal and Shelf Science, 1-14.

Warren, L.A., Tessier, A., Hare, L. (1998). Modelling Cd accumulation by benthic invertebrates in situ: the relative contributions of sediment and overlying water reservoirs to organism Cd concentrations. *Limnology and Oceanography*, 43 (7): 1442-1454.

Zar, J. (1999). *In*: Ryu T (ed) Biostatistical analysis, 4th edn. Prentice-Hall Inc., Upper Saddle River, 663 pp.

CHAPTER IV

Mercury concentration in mantle and digestive gland of common cuttlefish *Sepia officinalis* in a coastal lagoon with historical point-source discharges (Aveiro, Portugal)

IV. Mercury concentration in mantle and digestive gland of common cuttlefish *Sepia officinalis* in a coastal lagoon with historical point-source discharges (Aveiro, Portugal)

IV.1.1. Abstract

The mercury (Hg) levels were measured in mantle and digestive gland of common cuttlefish, *Sepia officinalis* collected from six sites (Barra (BAR), Torreira (TOR), Commercial Port (POR), Gafanha (GAF), Rio Novo do Príncipe (RIO), and Laranjo (LAR) in the Ria de Aveiro. The Hg concentrations were higher in Laranjo bay (1.2 and 2.0 µg g^{-1}, in mantle and digestive gland, respectively). In other areas the accumulation of Hg is much lower (0.25-0.65 µg g^{-1} in mantle and 0.40-1.20 µg g^{-1} in digestive gland). The results suggest that the effect of mercury contamination in cuttlefish is restricted to the Laranjo bay. Mercury in mantle and digestive gland were of the same order of magnitude, indicating that the digestive gland does not have a specific affinity to store this element. The 1.5 µg g^{-1} dry weight EU limit was only exceeded in one cuttlefish sample. It may concluded that the cuttlefishes studied do not constitute cause for concern in terms of human consumption, and could be safely used for daily intake of essential elements.

IV.1.2. Introduction

Mercury (Hg) is one of the most hazardous contaminants that may be present in aquatic environments, widely considered to be among the highest priority environmental pollutants in the scope of the European Water Framework Directive (WFD) and on the global scale (Pereira *et al.*, 2009a). Mercury is an element that has both natural and anthropogenic sources (OSPAR, 2004). Most of the anthropogenic mercury enters to aquatic ecosystems in its inorganic form through diffuse sources or via point discharges (Baeyers *et al.*, 2003). It is recognised that anthropogenic sources of mercury are responsible for the highest environmental impacts (EPA, 1997), having deleterious effects on biota, including both Human health and ecosystem functions (Pereira *et al.*, 2009a). Mercury is accumulated by many aquatic organisms, transferred and magnified along trophic chain (Baeyers *et al.*, 2003), eventually finding its way to economically important species and ultimately to humans (Pereira *et al,* 2006). Although, the existing restrictions on the anthropogenic sources of mercury, historically contaminated sediments may still constitute a source of mercury to the aquatic environment. In general, mercury has a high affinity with suspended particles which contributes to its removal from water and to sink in bottom sediments (Lee *et al.*, 1998; Ramalhosa *et al.*, 2001, Ramalhosa *et al.*, 2006). Thus, sediments may function as reservoir of anthropogenic mercury and/or as internal sources when metal is freed to pore-water (Arakel, 1995, May *et al.*, 1997, Baeyers *et al.*, 2003). Mercury retained in sediments may be released to the water column through physical disturbance as redox potential, temperature, oxygen, salinity, becoming available to aquatic organisms (Pereira *et al.*, 1998a, 1998b; Ramalhosa *et al.*, 2006; Pato *et al.*, 2008; Pereira *et al.*, 2009a). The integration of Hg may vary with different parameters such as biological (growth rate, size, sex) ecological (food, habit) and environmental factors (Hg availability, methylation rate, primary productivity) (Raimundo *et al,* 2010).

As several other cephalopod species, the common cuttlefish *Sepia officinalis* is extensively fished and consumed by humans of coastal communities in Portugal, considered important target specie in fisheries having a high economic, social and cultural value. Therefore, contaminant levels in cephalopods are also of direct concern to public health, since they could represent an important vector of contaminant to the consumers as shown for Cd (Bustamante *et al.*, 1998a).

Ria de Aveiro, an estuarine lagoon in the northwest of Portugal, is a biologically productive system with a significant role in the life cycle of several organisms being used as nursery for many species, namely cuttlefish. Over five decades, this marine system received discharges of anthropogenic mercury from a chlor-alkali plant (Pereira *et al.* 1998a, b; Pato *et al.*, 2008; Pereira *et al.*, 2009a). It is well known that chlor-alkali industries using mercury as a cathode are an important source of this hazardous contaminant in aquatic environments (Ramalhosa *et al.*, 2005). Since the influence of anthropogenic mercury in the environment persists for a substantial period of time after the reduction of emissions (Pereira, 1996), studies on the impact caused by emissions of mercury even after the sources have been extinguished are necessary. Mercury's high mobility, persistence in the environment, and lipophilicity, justify the importance of the environmental study of mercury, as it is a toxic element to all living organisms (Boening, 2000).

Several studies have been made to track the mercury discharges in the sediments (Lucas *et al.*, 1986; Hall *et al.*, 1985; Pereira *et al.*, 1998a; Ramalhosa *et al.*, 2001; Ramalhosa *et al.*, 2005; Pastorinho *et al.*, 2012); water (Hall, 1982; Pereira *et al.*, 1998b; Ramalhosa *et al.*, 2001; Pato *et al.*, 2008); organisms (Lima, 1986; Lucas *et al.*, 1986; Abreu *et al.*, 2000; Coelho *et al.*, 2006; Pereira *et al.*, 2006; Guilherme *et al.*, 2008) and plants (Coelho *et al.*, 2005; Abreu *et al.*, 2008). In contrast to fish or others organisms, information on Hg in cephalopod tissues is scarce (Raimundo *et al.*, 2004; Seixas *et al.*, 2005a,b; Bustamante *et al.*, 2006a; Pierce *et al.*, 2008; Pereira *et al.*, 2009), which contrast once more with the several studies concerning other trace metals in cephalopod tissues (*e.g.* Martin and Flegal, 1975; Smith *et al.*, 1984; Miramand and Bentley, 1992; Bustamante *et al.*, 2000, 2002a, b, 2004; Ayas and Ozogul, 2011). Almost all of these studies demonstrated the major role of digestive gland in metal metabolism, as this organ is deeply involved in assimilation processes, detoxification and storage of both essential and non-essential metals. Only two studies analysed the levels of Hg in digestive gland of common cuttlefish *Sepia officinalis* (Bustamante *et al.*, 2006a; Pereira *et al.*, 2009b).

Therefore, there is a need to provide more data on Hg concentration in digestive gland and edible tissue of cuttlefish to better estimate the intake of human populations feeding on this cephalopod. Thus, the present study aims at providing baseline data on total Hg concentration in the tissues (digestive gland and mantle) of the cuttlefish *S. officinalis* (L.) from six sites of the Ria de Aveiro lagoon, including areas with variable degrees of contamination. In addition to examining spatial and seasonal differences, biological data was also collected to present a contemporary picture of the distribution of size, maturity, and sex ratio.

IV.2. Materials and methods

IV.2.1.Study Area

The Ria de Aveiro (Figure IV.1), situated on the northern coast of Portugal and with a total area of 83 km^2 (high tide) and 66 Km^2 (low tide) (Dias *et al.*, 2001; Abrantes *et al.*, 2006), supports a population of 350,000 inhabitants (INE, 2001). Its principal municipaly (Aveiro town) is located 15 km south from the industrial complex of Estarreja, that includes a chlor-alkali plant that discharged mercury from the 1950s until the mid 1990s (Pereira *et al.*, 2009a). The estimated lagoon tidal prism ranges from 137 x 10^6 m^3 for maximum spring tide and 35 x 10^6 m^3 for minimum neap tide (Dias *et al.*, 2001). The total mean river input into the lagoon during tidal cycle is smaller (about 1.8 x 10^6 m^3) when compared with the tidal prism (Lopes and Dias, 2007). Consequently semidiurnal tides are the major factor influencing the hydrodynamics of the lagoon (Dias *et al.*, 2000). Laranjo bay is an internal source of Hg by the sediments that have been highly contaminated with Hg (Pereira *et al.*, 2009a). Dias (2001) and Lopes *et al.* (2006) characterized the first half of the main channels of the Ria de Aveiro lagoon as ebb dominated and the second half as flood dominated. As the lower lagoon is ebb dominated there is a trend to export sediments to the ocean (Picado *et al.*, 2010). Taking this in consideration and the several sources of pollution of the lagoon, the following

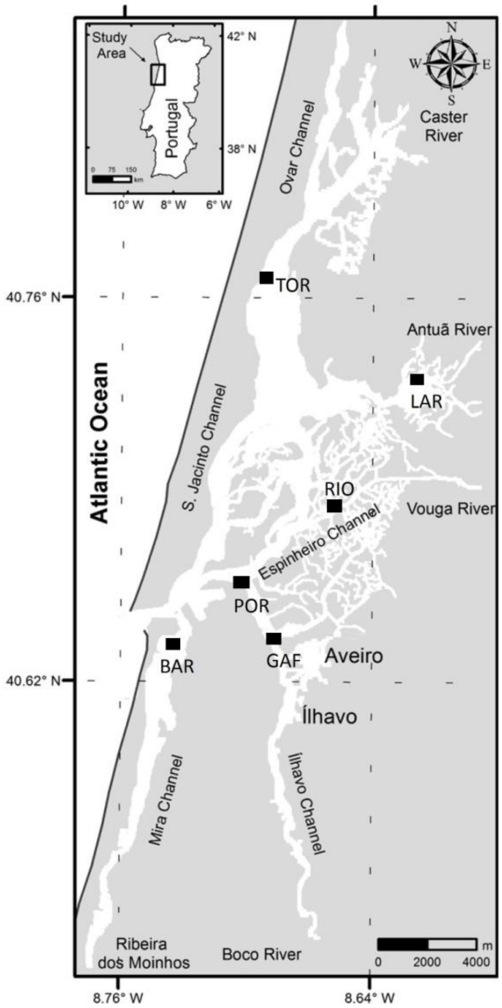

Figure IV.1- Sampling sites of *S. officinalis*: Ria de Aveiro (Adapted from Valentim *et al.*, 2013).

sites were selected: Barra (BAR), near the entrance of the lagoon in the initial part of the Mira channel and subject to considerable vessel traffic; Commercial Port (POR), within the harbour and so with the influence of all the activities developed in the harbour; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also connected with the main channel coming from Aveiro city carrying domestic discharges; Torreira (TOR), an intermediate region of the longest channel (S. Jacinto-Ovar channel) with influence of Largo da Coroa, considered the second most contaminated area after Laranjo bay in Ria de Aveiro; Rio Novo do Principe (RIO) located in the terminal area of the main freshwater course, the Vouga river, 6.5 km distant from a paper mill effluent outlet; Laranjo (LAR), in the proximity of the chlor-alkali plant (6 km), the principal source of metal contamination (mainly mercury).

IV.2.2.Sampling and biological data collection

Fifty three common cuttlefish, *S. officinalis* (Table IV-I), were caught from March to December 2003, with a fishing net, a network trammel, in five sites of Ria de Aveiro (Figure IV.1). The abiotic parameters such as temperature, dissolved oxygen, pH, salinity, turbidity and depth were registered on each capture. Temperature (± 0.1 ºC) and dissolved oxygen (± 0.01 $mg.l^{-1}$) were recorded with an oxygen meter (Consort Z621), the pH (± 0.01) with pH meter (*WTW* 330/set – 2), the salinity (± 0.1) with a refractometer (*Atago*), the turbidity (± 0.1 m) was recorded with a Secchi disc and the depth (± 0.1m) with a handmade probe. Total body weight, dorsal mantle length (DML, size), total length (TL), sex and maturity stage were determined in each individual. All individuals were included in an allometric relationship between fresh weight (g) and mantle length (mm) (Figure IV.2). A standard 5-point scale of sexual maturity for *S. officinalis* was used (adapted from Alonso-Allende and Guerra, 1984) (Table A. 1 - Appendix), in which stage I is “immature”, stages II-III are “maturing”, and stages IV-V are “mature”. Specimens with no visible gonad development were classified as “Juveniles” (Challier *et al.*, 2002) and allocated in maturity stage 1. The origin, number of individuals and body weight of each specimen are given in Table IV-I. Specimens were stored in individual plastic bags and frozen (-80 ºC) in order to minimize mobilization of metals between organs (Martin and Flegal, 1975). In the laboratory, digestive gland and mantle (without skin) were totally removed under partially defrost conditions without rupture of the tissues. Weight of digestive gland was expressed as percentages of total body weight, *e.g.*, digestive gland index (DGI, see Jorge and Sobral, 2004). After separation, individual tissue samples were freeze-dried, ground and homogenised for the analysis of total mercury.

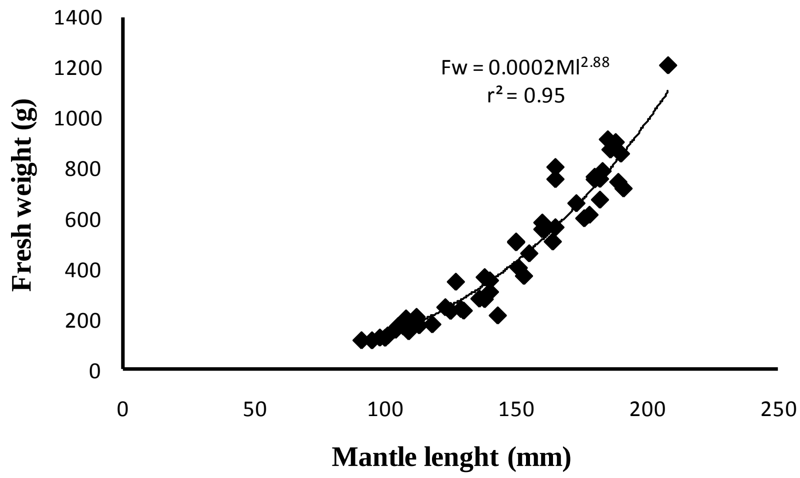

Figure IV.2 – Allometric relation between total fresh weight (g) and mantle length (mm) of the *S. officinalis* captured in the Ria de Aveiro.

Table IV-I – Characteristics of sampled cuttlefishes.

Sampling area	Sample size	Mantle length (mm)	Total weight (g)	Sex	Stage of maturation
BAR	9	134.3±31.0 (98-187)	348.6±261.8 (126.3-894.7)	7♂, 2♀	II-1; III-3; IV-5
GAF	5	129.0±39.0 (91-189)	299.5±258.8 (115.6-745.4)	5♂	II-1; III-2; IV-2
LAR	3	156.0±25.2 (140-185)	479.1±380.3 (214.4-914.9)	1♂, 2♀	III-1; IV-2
POR	25	158.0±32.3 (95-208)	563.8±278.7 (115.3-1209.5)	13♂, 12♀	II-4; III-4; IV-17
RIO	3	111.0±3.0 (108-113)	195.4±17.3 (175.1-208.5)	3♂	IV-3
TOR	8	144±27.1 (105-183)	436.8±255.8 (172.1-804.6)	5♂, 3♀	III-2; IV-6

* mean ± standard deviation. Values between brackets represent the interval for the variable

IV.2.3. Analytical procedures

Total Hg was determined by atomic absorption spectrometry, using a silicon UV diode detector Leco AMA-254, after pyrolysis of each sample in a combustion tube at 750 ºC, under an oxygen atmosphere and collection on a gold amalgamator (Costley *et al.*, 2000). Mercury analysis were ran with respect to a methodical quality control including analysis of international certificate standards dogfish muscle DORM-1, DORM-2; lobster hepatopancreas TORT-1, TORT-2 and fish liver DOLT-3 purchased to the National Research Council, Canada. These certified materials were treated and analysed under the same conditions as the samples. The results were in good agreement with the standards values, not differing significantly at 95 % confidence level (Table IV-II). Blanks were also run together with samples. Concentration of metal was expressed in dry weight (μg g^{-1} DWT). Detection limit for Hg was 0.041 μg g^{-1}.

Table IV-II – Concentration of Hg in standard materials used to accuracy analytical quality control (mean±SD; μg g^{-1} dry weight)

Standard tissue		Hg (μg g^{-1})
DORM-1	Present study	0.80±0.016
	Certified	0.80±0.074
DORM-2	Present study	4.50±0.240
	Certified	5.20±0.650
TORT-1	Present study	0.32±0.007
	Certified	0.33±0.060
TORT-2	Present study	0.29±0.005
	Certified	0.27±0.060
DOLT-3	Present study	3.24±0.030
	Certified	3.37±0.140

IV.2.4. Data analysis

Specific weights of metal concentrations were calculated as a function of dry weight. Prior to statistical analysis, Hg concentrations and biological data were tested for normality and equality of variance using the Anderson-Darling test and Levene´s test, respectively. Whenever significant deviations from normality and homoscedasticity were found, data was log-transformed prior to further statistical analysis to correct for these deviations. For Hg, statistical comparisons between sites, sex and tissues were performed using a two-way ANOVA followed by post hoc Tukey comparisons whenever significant differences were found. Pairing site and season was also used to assess seasonality effects using a two way ANOVA. For relations between other parameters such as mantle length, total weight, DGI, and concentration of Hg, was used Pearson coefficient of correlation. Statistical testing was carried out with MINITAB® Release 14.20. A significance level of 0.05 was used for all tests. Redundancy Analysis (RDA), a technique that falls between multiple regression and canonical correlation analysis (Sparks *et al*. 1999), was performed using the CANOCO software package for windows, version 4.5 to assess effects of environmental conditions on metal content. The significance of RDA was tested using a Monte Carlo permutation test using routines built into the software package. A detailed description of the method is provided in Van den Brink *et al.* (2003).

IV.3. Results

IV.3.1. Metal in mantle and digestive gland

The differences in Hg concentrations tended to be slightly higher in the digestive gland than in mantle (Table IV-III). Despite the small difference between Hg levels in both tissues (values of the same order of magnitude), the between-tissues difference in concentration was significant for all areas of sampling ($F_{1, 99}$=63.59, p<0.001).

Table IV-III - Number of specimens and concentrations of Hg (mean±SD, µg g^{-1} DWt) by sampling site in tissues (mantle and digestive gland) of *S. officinalis*.

Site	n	Hg	
		Mantle	Digestive gland
BAR	9	0.41±0.064	0.51±0.072
		(0.34-0.51)	(0.43-0.60)
GAF	5	0.49±0.15	0.87±0.28
		(0.33-0.65)	(0.60-1.20)
LAR	3	1.20±0.42	2.00±0.68
		(0.91-1.64)	(1.53-2.80)
POR	25	0.40±0.10	0.64±0.18
		(0.25-0.60)	(0.40-1.11)
RIO	3	0.40±0.054	0.68±0.091
		(0.32-0.43)	(0.60-0.80)
TOR	8	0.41±0.091	0.51±0.098
		(0.31-0.52)	(0.41-0.67)

*Values between brackets represent the interval for the variable

Two-way ANOVA was used to show significant differences in metal concentration between site, sex and season for mantle and digestive gland (Table IV-IV). Highly significant ($p<0.001$) site-specific differences were detected in both tissues. The lagoon can be divided in two different areas in terms of Hg concentrations: a larger group without differences which includes the sites of BAR, POR, TOR, RIO and GAF, that can be considered as the area with lower Hg concentrations and a contaminated area, LAR. For sex only mantle presented significant differences ($p<0.001$), in this case females presented higher values of Hg accumulated. In the seasonal analysis, only the digestive gland presented significant differences of Hg concentrations (Table IV-IV). In this tissue, all the sampling sites presented higher levels in summer than in spring.

Table IV-IV – Results of two-way ANOVA for effects of site, sex and season on mercury concentrations in all tissues analysed followed by conclusions from post hoc testing using the Tukey test.

Mantle			
Factors	F	Significance (p value)	Conclusions
Site	$F_{5,47}=10.49$	<0.001 (S)	BAR=RIO=POR=TOR=GAF≠LAR
Sex	$F1_{,46}=18.13$	<0.001 (S)	F≠M
Season	$F_{1,46}=0.31$	0.578 (NS)	-
Digestive Gland			
Factors	F	Significance (p value)	Conclusions
Site	$F_{5,47}=8.56$	<0.001 (S)	BAR=TOR=POR=RIO=GAF≠LAR
Sex	$F1_{,46}=1.17$	0.197 (NS)	-
Season	$F_{1,46}=5.10$	0.029 (S)	spring≠summer (*)

(*) comparison not conclusive for autumn and winter

The effect of body size (DML) which ranged for 91-208 mm was also evaluated, however the result was non-significant for both tissues analysed ($p<0.05$).

Correlations of Hg in tissues

The Hg concentrations in mantle and digestive gland presented a positive linear correlation (Pearson correlation of 0.69 with a $p<0.001$), so higher values of Hg in digestive gland corresponded to higher values of Hg in mantle (Figure IV.3). The positive correlation between Hg levels in mantle and digestive gland is independent of the sampling site (Figure IV.4). Taking into account the low number of specimens analysed, the regression between Hg concentrations in the two tissues is strongly significant. When cuttlefish is subjected to lower concentrations of Hg the partition of this element to the mantle and digestive gland is almost the same. However, when the levels of Hg increase, the importance of the digestive gland in the storage of several metals seems to become effective (Miramand and Guary, 1980; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante *et al*., 2002a, b, 2004) (Figure IV.4).

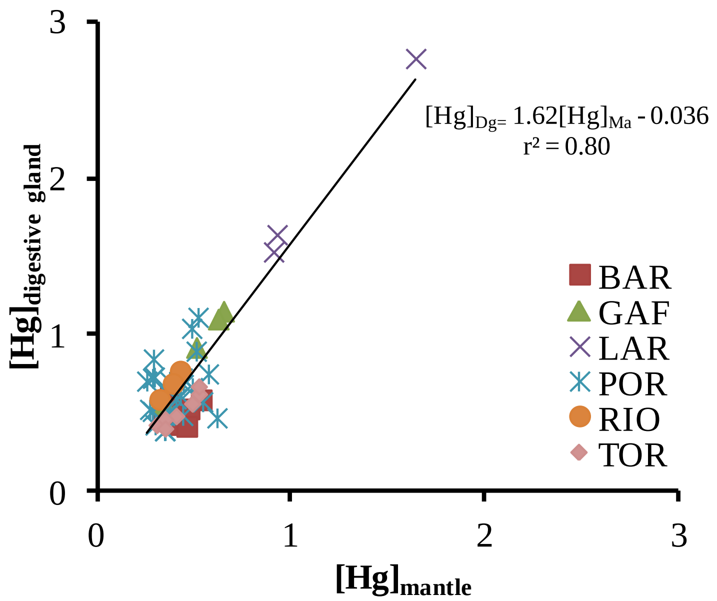

Figure IV.3 – Relationships between concentrations of Hg (µg g^{-1}DWt) in mantle and digestive gland in *S. officinalis* from Ria de Aveiro.

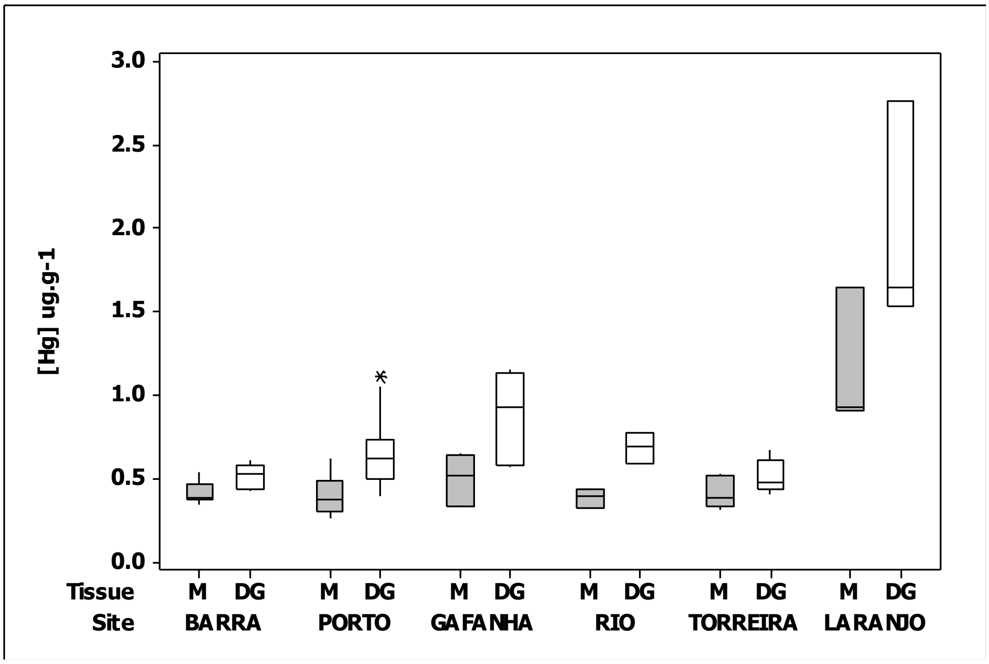

Figure IV.4 – Boxplot of Hg levels (µg g^{-1}DWt) for tissue and site.

IV.3.2.Implications for public health

According to COMMISSION REGULATION (EC) Nº 629/2008 of 2 July 2008, the limit established for the Hg content in the mantle of cephalopods is 0.5 µg g^{-1} (fresh weight) of Hg. Moreover, Portugal has also signed the OSPAR convention, in which it was established that the maximum level of mercury in animals for human consumption is 0.3 µg g^{-1} wet weight (i.e., 1.5 µg g^{-1} dry weight) (Seixas *et al.*, 2005b).

Considering average of Hg levels in mantle of cuttlefish, the values are significantly lower than the EU limit (1.5 µg g^{-1} DWt) (Figure IV.5). Taking into account all the results obtained it was found that only one individual from Laranjo presented a value higher (1.6 µg g^{-1} DWt) than the limit recommended for human consumption (Figure IV.5).

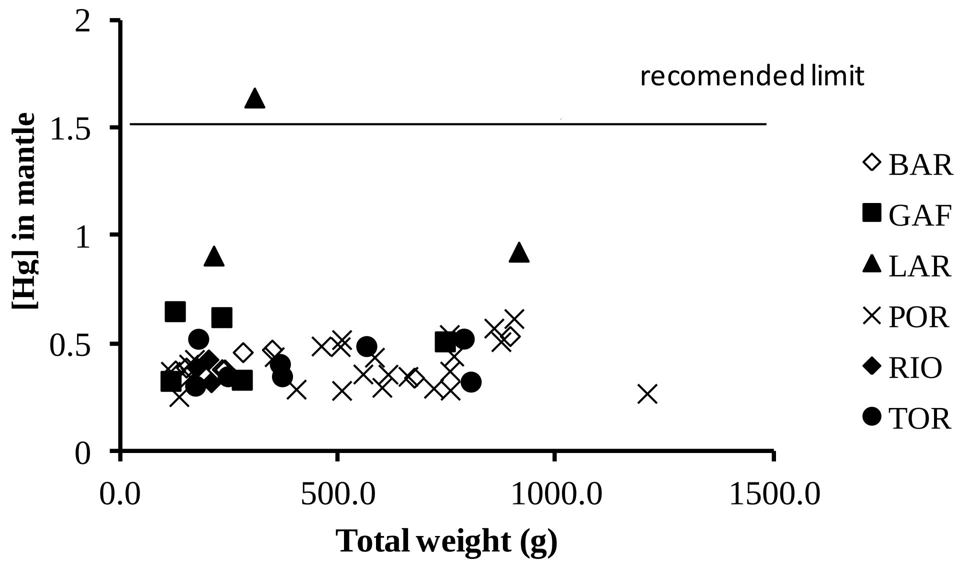

Figure IV.5 – Comparison between Hg concentration (μg g^{-1} DWt) in mantle and the recommended limit for human consumption, from different sampling areas.

Considering the body size of the analysed specimens the ingestion of the digestive gland is not suppose to occur, for this reason the values obtained for these tissue weren't compared with the EU limit for human consumption.

IV.3.3.Redundancy Analysis (RDA)

RDA analysis showed that most of the variance is associated with the first axis (97.4%), and the remaining part is explained by the second axis (2.6%). Monte Carlo permutation tests followed the RDA and showed significance for the first axis ($p=0.006$), thus, differences found in the first axis are significant (Figure IV.6). The significance test of all canonical axes was also statistically significant ($p=0.006$).

The results of multivariate analysis are comparable to the univariate one (ANOVA), however, the results of the multivariate analysis show more details in individual levels of Hg in tissues. The RDA focus on the absolute differences in 'species' abundances (here Hg levels) between the 'samples' (here sites). By the analysis of the RDA triplot is easily understood that LAR presents the higher values of Hg concentrations and the rest of the lagoon lower values of Hg concentrations. In terms of environmental parameters the factor that most interfere with the Hg concentration is the temperature. It seems that temperature makes the distinction between LAR and the rest of the lagoon. Temperature is strongly correlated with the first axis (Figure IV.6).

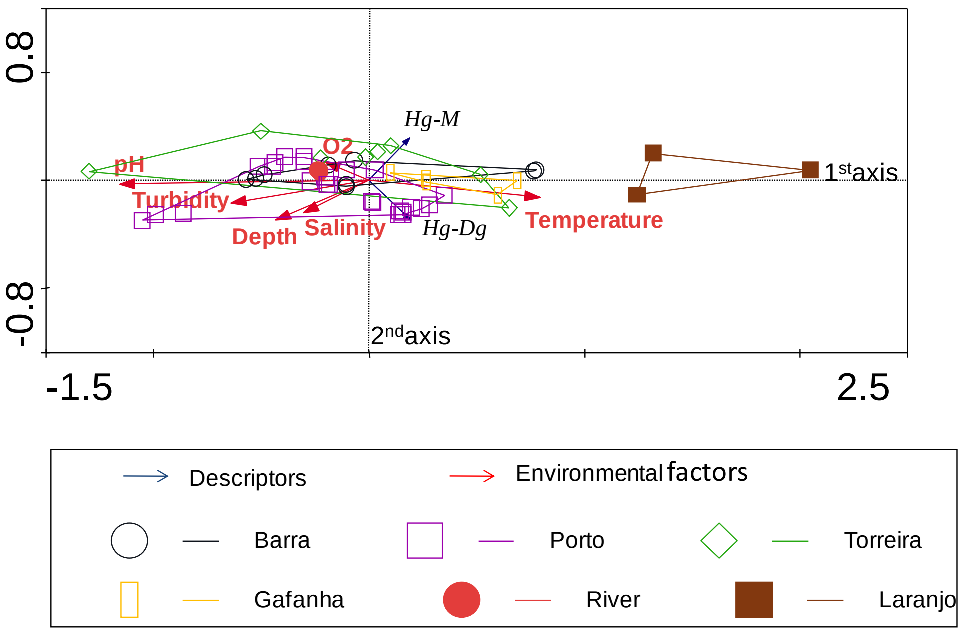

Figure IV.6 – RDA triplot, with focus on site sampling, sampling tissues and environmental parameters.

IV.4. Discussion

Univariate and multivariate methods used in this work are concordant in terms of Hg distribution in Ria de Aveiro, and levels of Hg are low with the exception of Laranjo bay that presented higher values. This fact eliminates a regional distribution of Hg in Ria de Aveiro, the higher levels of Hg are certainly related with the historical discharges of effluents from the chlor-alkali industry of the industrial complex of Estarreja into Laranjo bay (Lucas *et al.*, 1986; Hall *et al.*, 1985; Pereira *et al.*, 1998a; Reis *et al.*, 2009; Monterroso *et al.*, 2003; Pereira *et al.*, 2009a). Today Laranjo bay is a non-significant active source of Hg since the discharges stopped in 1985, however, it is still an internal source of Hg by the sediments that have been highly contaminated with Hg (Hall *et al.*, 1985). The Hg contained in the sediments may became available to biota (Coelho *et al.*, 2007), eventually from food chain by the benthic organisms (Bryan and Langston, 1992; Lee *et al.*, 1998; Warren *et al.* 1998), or be remobilized when sediments are dredged and disposed into water bodies (Newell *et al.*, 1998). The RDA analysis clearly separates Laranjo area from the rest of the lagoon, and temperature is the factor that most influences this distribution (Figure IV.6). Laranjo presented higher values of temperature than other sites, which probably influenced the Hg concentrations, since high temperature favors a high methylation rate (Bustamante *et al.*, 2006a), which could lead to a higher uptake of mercury by aquatic organisms. Other authors also referred that environmental condition, such as levels in water, exposure period, and temperature may influence the metal accumulation in cephalopods (Bustamante *et al.*, 1998b, 2000).

The cuttlefish *Sepia officinalis* feeds on a large variety of living prey, including small molluscs, crabs, shrimps, other cuttlefish, and juvenile demersal fishes. Schuhmacher *et al.* (1994) concluded that the groups that accumulated more Hg are crustaceans and fishes, that are preferential preys of Cuttlefishes (Pinczon du Sel *et al.*, 2000) Taking into account the cephalopods capacity to bioaccumulation (Bustamante *et al.* 1998b, 2000, 2006a) and that cephalopods have economic importance for humans, the analyse of Hg levels in Ria de Aveiro is relevant. The levels of mercury in this study were in general below the acceptable limit for human consumption, only one value of Laranjo bay was higher than the limit (see Figure IV.5). Levels of Hg obtained in this study show that this specie does not constitute a cause of concern in terms of human consumption. However, due to the low number of individuals captured in Laranjo bay and the constant concern with human health, further studies in this area are needed for a better evaluation of the problem.

Mercury is considered to be one of the most problematic metals in marine ecosystems due to bioaccumulation and biomagnification in marine food webs (EPA, 2001). Various studies reported Hg concentrations in different tissues (Alcobia, 1995; Buzina *et al.*, 1989; Stoeppler *et al.*, 1979; Rossi *et al.*, 1993; Seixas *et al.*, 2005a; Raimundo *et al.*, 2004, 2010; Bustamante *et al.*, 2008). Table IV-V compares Hg concentrations in mantle and digestive gland of cuttlefish with other cephalopods species. The values obtained revealed two distinct areas in Ria de Aveiro, the Laranjo bay (polluted area) with high concentration of Hg, and the rest of the lagoon as a whole, presenting low concentration of Hg. Considering the specie *S. officinalis*, and comparing only the polluted areas, the value presented for Laranjo bay is higher than the value of Kastela bay (polluted). When analysing the unpolluted areas of Ria de Aveiro the value obtained is higher than those presented on the referred studies. (Table IV-V) Mercury concentrations in the lagoon are greatly influenced by the historical discharges of mercury from industrial activities into Laranjo Bay (Pereira *et al.*, 1998a; Pereira *et al.*,

2009), leading to Hg concentrations higher than other ecosystems also polluted like Kastela Bay in Adriatic that is known for obtaining discharges of Hg from mine exploitation of the region (Faganeli *et al.,* 2003).
Considering different species, Hg concentrations in this work were always higher with the exception of the species (*Eledone cirrhosa* and *Octopus vulgaris*) captured in Adriatic Sea, Mediterranean Sea and specific areas in Portuguese Coast (Cascais and Olhão). Higher Hg concentrations in Mediterranean organisms are typically explained by high temperature and the absence of solar radiation in the deep environment that favours a high methylation rate (Bustamante *et al.*, 2006a). Moreover, natural sources of Hg in the Mediterranean Sea may contribute to Hg enrichment through the benthic food webs, as it constitutes the richest natural reserve of this element (Bacci, 1989 in Bustamente *et al.,* 2006a). Geological characteristic such as sulphide deposits of the Iberian Pyrite Belt (Leistel *et al.*, 1998) affect the Hg concentrations of the Gulf of Cadiz (Cossa *et al.,* 2001). Taking into account the mainstream of the Mediterranean Sea and as a result of cyclonic currents in the eastern shelf of the South coast of Portugal the availability of Hg to local food web can be increased (Garcia-Lafuente and Ruizet, 2006; Relvas *et al.,* 2007). The octopus is a top predator (Mangold, 1983) and this could justify the higher concentrations of mercury. These facts could explain the higher values obtained for *Eledone Cirrhosa* and *Octopus vulgaris* when compared with cuttlefish of the northwestern coast of Portugal. Like other metals, Hg concentrations in cephalopod may vary with biological factors such as age (size), sex and maturity stage (Bustamante *et al.*, 1998a; Raimundo *et al.,* 2004; Seixas *et al.*, 2005a; Ayas and Ozogul, 2011). It is generally admitted that Hg concentrations in cephalopod tissues are positively correlated with size (Monteiro *et al.*, 1992; Rossi *et al.,* 1993; Storelli and Marcotrigiano, 1999 *in* Bustamante *et al.,* 2006a), however due to restrict range of size of captured individuals this couldn’t be verified. It would be extremely interest to invest more studies in Ria de Aveiro that includes a higher range of sizes, in order to verify this effect of bioaccumulation along life cycle of cuttlefish.

Table IV-V - Mean ±SD (µg g^{-1} DWt) of Hg or Hg concentrations (µg g^{-1} DWt) in mantle and digestive gland of cephalopods from the literature.

Species	Sites	Mantle	Digestive gland	Authors
Sepia officinalis	Ria de Aveiro (Portugal)	0.41±0.10	0.66±0.29	Present study
	Laranjo Bay, Ria de Aveiro	1.16±0.42	1.98±0.68	Present study
	Sado estuary (Portugal)	0.10	0.20	Alcobia, 1995
	Kastela Bay (Adriatic) unpolluted	0.24	-	Buzina et al., 1989
	Kastela Bay (Adriatic) polluted	0.48	-	Buzina *et al*., 1989
	La Spezia (Mediterranean)	0.34	-	Stoeppler *et al*., 1979
	Maddalena (Mediterranean)	0.20	-	Stoeppler *et al*., 1979
	Chioggia	0.16	-	Stoeppler *et al*., 1979
	Ceuta	0.13	-	Stoeppler *et al*., 1979
	Scheveningen	0.08	-	Stoeppler *et al*., 1979
Architeuthis dux	Bay of Biscay	-	0.47±0.13	Bustamante *et al*., 2008
	Mediterranean	-	1.56	Bustamante *et al*., 2008
Eledone Cirrhosa	Mediterranean Sea	1.00-6.02	-	Rossi *et al.*, 1993
Loligo vulgaris	Kastela Bay (Adriatic) unpolluted	0.26	-	Buzina *et al*., 1989
	Kastela Bay (Adriatic) polluted	0.32	-	Buzina *et al*., 1989
Octopus vulgaris	Adriatic Sea	2.70±1.20	1.50±1.0	Storelli *et al*., 2006
	Viana (Portugal)	0.27±0.04	0.58±0.08	Seixas *et al*.,2005a
	Cascais (Portugal)	0.48±0.16	3.43±2.57	Seixas *et al*.,2005a
	Portuguese coast	0.31±0.12	2.60±4.30	Raimundo *et al.*, 2004
	Matosinhos (Portugal)	0.28±0.14	0.86±0.54	Raimundo *et al.*, 2010
	Cascais (Portugal)	0.60±0.20	1.80±1.10	Raimundo *et al.*, 2010
	Olhão (Portugal)	0.35±0.04	5.10±1.50	Raimundo *et al.*, 2010
	Kastela Bay (Adriatic)	0.52	-	Buzina *et al*., 1989
	Mediterranean Sea	0.44-1.38	4.61-60.56	Renzoni *et al*.,1973

The distribution of Hg was considered between two separated tissues, mantle (muscle) and digestive gland of *S. officinalis.* Mantle muscle was first selected taking into account that it is referred by several authors (*e.g.* Renzoni *et al.,* 1973; Monteiro *et al.,* 1992; Seixas *et al.,* 2005a) as a body compartment that could store Hg in significant amounts and also for human health purpose. The second organ, digestive gland, plays a major role in storage and detoxification of several metals. In contrast to what was observed for other trace metals, like Ag, Cu, Zn and Cd in previous studies (*e.g.* Miramand and Guary, 1980; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante *et al.*, 2002a, b, 2004) where concentrations in digestive gland were one or more orders of magnitude higher than in other tissues, for Hg the concentrations recorded for digestive gland were in the same order of magnitude compared to the mantle. Such different patterns of accumulation between trace metals and Hg suggest that cephalopods have different mechanisms/rates of uptake and/or sequestration of these metals (Pierce *et al*., 2008). This contrast between trace metals and Hg partitioning was also observed in other cephalopods (Raimundo *et al.*, 2004; Seixas *et al.*, 2005a; Bustamante *et al.*, 2006a).

Sources of metal contamination in cephalopods are seawater, as it passes through the skin and through the gills; and diet, which probably represents the main pathway for many elements, as previously shown for Cd and Zn (Bustamante *et al.*, 2002b, 2004, 2006b; Miramand *et al.*, 2006). Despite the comparable concentration of Hg, the digestive gland presented in all sites a higher value than mantle (see Table IV-III), which strongly suggests that food is a major source for mercury accumulation in cuttlefish. Due to the positive correlation between Hg concentrations in mantle and digestive gland (Figure IV.3), and the fact that muscular tissues contained most of the body's burden of mercury, which suggests a translocation of Hg from digestive gland (Seixas *et al.*, 2005a; Lacoue-Labarthe *et al.*, 2009). The small difference between levels of Hg in mantle and digestive gland could be explained by the affinity of mantle to storage MeHg, that is the predominant form of Hg stored in muscle tissue (Harris *et al*., 2003; Amlund *et al*., 2007). However, compared to other metals like Cd, the role of digestive gland in storage of Hg appears to be relatively limited. This may be due to an excretion function of Hg by the digestive gland (Lacoue-Labarthe *et al.*, 2009), and a preferential redistribution of Hg to muscular tissues where it bound to the sulfhydryl groups of proteins (Bustamante *et al*., 2006a).

IV.5. Conclusions

The study provided new data on one of the most problematic contaminant in marine ecosystems, mercury, in edible tissue and digestive gland of the commonly eaten *S. officinalis* from Portugal, and clearly demonstrated that levels of mercury in Ria de Aveiro lagoon do not constitute a risk to human health. Actually the increment of temperature concentrated the contaminants in the aquatic environment and the known presence of historical anthropogenic discharges of mercury from a chlor-alkali plant near this site lead to a higher accumulation of Hg in *S. officinalis* specie in Laranjo bay. However, further and wider scale study would be valuable, especially in a restricted polluted area, Laranjo. Taking into account that organic form of Hg is the most toxic, it would be interesting in future studies to measure MeHg, when aiming to evaluate the risk for human consumption. In the same way, supplementary analysis concerning Hg levels are needed to really understand the apparent limited role of digestive gland in storage and detoxification of this element when compared with others trace elements like Cd and Zn. Observed seasonal and spatial variations indicate that cuttlefish can be used to monitor the Hg levels in the environment.

IV.6. References

Abrantes, I., Dias, J., Rocha, F. (2006). Spatial and temporal variability of suspended sediments concentration in Ria de aveiro and fluxes between the lagoon and the ocean. *Journal of Coastal Research*, 39: 718-723.

Abreu, S.N., Pereira, E., Vale, C., Duarte, A.C. (2000). Accumulation of Mercury in Sea Bass from a Contaminated Lagoon (Ria de Aveiro, Portugal). *Marine Pollution Bulletin*, 40(4): 293-297.

Abreu, A.N., Soares, A.M.V.M., Nogueira, A.J.A., Morgado, F. (2008). Tree Rings, *Populus nigra* L., as Mercury Data Logger in Aquatic Environments: Case Study of an Historically Contaminated Environment. *Bulletin of Environmental Contamination and Toxicology,* 80: 294-299.

Alcobia, H. (1995) Biologia e acumulação de mercúrio no choco *Sepia officinalis* (Linnaeus, 1758) do Estuário do Sado. Relatório de estágio de licenciatura. IPIMAR and FCUL, Lisboa, 46p.

Alonso-Alende, J.M. and Guerra, A. (1984). Crustáceos decápodeos y moluscos de la ria de Vigo (mimeo). Presentado alos prémios de investigación 1984, Excma. Diputación de Pontevedra, 152 p.

Amaratunga, T. (1983). The role of cephalopods in the marine ecosystem. In I.F. Caddy, *Advances in assessment of world cephalopod resources*. *FAO Fisheries Technical Paper*, 231: 379-415.

Amlund, H., Lundebye, A.-K., Berntssen, M.H.C. (2007). Accumulation and elimination of methylmercury in Atlantic cod (*Gadus morthua* L.) following dietary exposure. *Aquatic toxicology*, 83: 323-330.

Arakel, A.V. (1995). Towards developing sediments quality assessment guidelines for aquatic systems: an Australian perspective. *Australian Journal Earth Sciences* 42: 335-369.

Ayas, D. and Ozogul, Y. (2011). The effects of Season and sex in the Metal Levels of Mature Common Cuttlefis (*Sepia officinalis*) in Mersin Bay, Northeastern Mediterranean.*Journal of food Science*, Vol.76:4.

Bacci, E. (1989). Mercury in the Mediterranean. *Marine Pollution Bulletin,* 20: 59-63.

Baeyers, W., Leermakers, M., Pappina, T., Saprykin, A., Brion, N., Noyen, J. *et al*. (2003). Bioconcentration and biomagnifications of mercury and methylmercury in the North Sea and Scheldt estuary fish. *Archives of the Environmental Contamination and Toxicology*, 45, 157-163.

Boening, D.W. (2000). Ecological effects, transport, and fate of mercury: a general review. *Chemosphere*, 40: 1335-1351.

Bryan, G.W., Langston, W.J. (1992). Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuarines: a review. *Environmental Pollution,* 76: 89-131.

Bustamante, P., Caurant, F., Fowler, S., Miramand, P. (1998a) Cephalopods as a vector for the transfer of Cd, to top marine predators in the North-East Atlantic Ocean. *Science of the Total Environment*, 220: 71-80.

Bustamante, P., Cherel, Y., Caurant, F., Miramand, P. (1998b) Cd, copper and Zn in octopuses from Kerguelen Islands, Southern Indian Ocean. *Polar Biology*, 19: 264-271.

Bustamante, P., Grigioni, S., Boucher-Rodoni, R., Caurant, F., Miramand, P. (2000). Bioaccumulation of 12 trace elements in the tissues of Nautilus *Nautilus macromphalus* from New Caledonia. Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environmental*, 368: 585-596.

Bustamante, P., Cosson, R.P., Gallien, I., Caurant, F., Miramand, P. (2002a). Cadmium detoxification processes in the digestive gland of cephalopods in relation to accumulated Cd concentrations. *Marine Environmental Research*, 53: 227-241.

Bustamante, P., Teyssié, J., Fowler, S.W., Cotret, O., Danis, B., Miramand, P., Warnau, M. (2002b). Biokinetics of zinc and cadmium accumulation and depuration at different stages in the life cycle of the cuttlefish *Sepia officinalis*. *Marine Ecology Progress Series*, 231: 167-177.

Bustamante, P., Teyssié, J.-L., Danis, B., Fowler, S.W., Miramand, P., Cotret, O., Warnau, M. (2004). Uptake, transfer and distribution of silve rand cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. *Marine Ecology Progress Series*, 269:185-195.

Bustamante P., Lahaye V., Durnez C., Churlaud C., Caurant F. (2006a). Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environmental*, 368: 585-596.

Bustamante, P.,Teyssié, J.L., Fowler, S.W., Warnau, M. (2006b). Assessment of the exposure pathway in the uptake and distribution of americium and cesium in cuttlefish (*Sepia officinalis*) at different stages of its life cycle. *Journal of Experimental Marine Biology and Ecology*, 331: 198-207.

Bustamante, P, González, A. F., Rocha, F., Miramand, P., Guerra, A. (2008). Metal and metalloid concentrations in the giant squid *Architeuthis dux* from Iberian Waters. *Marine Environmental Research*, 66,:278-287.

Buzina, R., Suboticanec, K., Vukusic, J., Sapunar, J., Antonic, K., Zorica, M. (1989). Effect of industrial pollution onseafood contente and dietary intake of total and methylmercury. *Science of the Total Environment*, 78: 45-57.

Challier, L., Royer, J., Robin, J-P. (2002). Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. *Aquatic Living Resources*, 15: 303-311.

Coelho, J.P., Pereira, M.E., Duarte, A., Pardal, M.A. (2005). Macroalgae response to a mercury contamination gradient in a temperate costal lagoon (Ria de Aveiro), Portugal). *Estuarine, Coastal and Shelf Science*, 65: 492-500.

Coelho, J.P., Rosa, M., Pereira, E., Duarte, A., Pardal, M.A. (2006). Pattern and annual rates of *Scrobicularia plana* mercury bioaccumulation in a human induced mercury gradient (Ria de Aveiro, Portugal). *Estuarine, Coastal and Shelf Science*, 69: 629-635.

Coelho, J.P., Policarpo, E., Pardal, M.A., Millward, G.E., Pereira, M.E., Duarte, A.C. (2007). Mercury contamination in invertebrate biota in a temperature coastal lagoon (Ria de Aveiro, Portugal). *Marine Pollution Bulletin*, 54: 464-488.

COMMISSION REGULATION (EC) Nº 629/2008 of 2 July 2008 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, L 173/6.

Costley, C.T., Mossop, K.F., Dean, J.R., Garden, L.M., Marshall, J., Carrol, J. (2000). Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation. *Analytica Chimica Acta*, 405: 179-183.

Cossa, D., Elbaz-Poulichet, F., Nieto, J.M. (2001). Mercury in the Tinto-Odiel estuarine system (Gulf of Cádiz, Spain): sources and dispersion. *Aquatic Geochemistry,* 7(1): 1-12.

Dias, J., Lopes, J., Dekeyser, I. (2001). Lagrangian transport of particles in Ria de Aveiro lagoon, Portugal. *Physics Chemistry Earth Part B: Hydrology Oceans Atmosphere*, 26: 721-727.

Dias, J.M. (2001). Contribution to the study of the Ria de Aveiro hydrodynamics, PhD thesis, Universidade de Aveiro, Portugal, pp.288.

EPA – United States Environmental Protection Agency. (1997). Fate transport of mercury in environment. In: Mercury study report to congress (Vol.III). EPA – 452/R-97-005.

EPA – United States Environmental Protection Agency. (2001). Water quality criterion for the protection of human health: methylmercury. *Office Science and Technology, Office of Water.* Washington, pp. 253.

Faganeli, J., Horvat, M., Covelli, S., Fajon, V., Logan, M., Lipej, L., Cermelj, J. (2003). Mercury and methylmercury *in* the Gulf of Trieste (Northern Adriatic Sea). *Science of the Total Environmental*, 304: 315-326.

Finger, J.M, Smith, J.D. (1987). Molecular association of Cu, Zn, Cd and ^{210}Po in digestive gland of the squid *Nototodarus gouldi. Marine Biology,* 95: 87-91.

Garcia Lafuente, J., Ruizet, J. (2006). The Gulf of Cádiz pelagic ecosystem: a review.

Progress *in Oceanography*,74 (2-3): 228-251.

Guilherme, S., Válega, M., Pereira, M.E., Santos, M.A., Pacheco, M., (2008). Antioxidant and biotransformation responses in Liza aurata under environmental mercury exposure—relationship with mercury accumulation and implications for public health. *Marine Pollution Bulletin*, 56: 845–859.

Hall, A. (1982). Water quality problems in Ria de Aveiro. In: Actual Problems of Oceanography in Portugal, Junta Nacional de Investigação científica e Tecnológica and Nato Marine Sciences Panel (ED.), Lisboa, pp.159-169.

Hall, A., Lucas, M.F., Caldeira, M.T., Duarte, A.C. (1985). Distribuição de Mercúrio nos Sedimentos da Ria de Aveiro (Distribution of Mercury in the sediments of Ria de Aveiro, Portugal). Actas das jornadas da Ria de Aveiro. Poluição da Ria de Aveiro, I. Câmara de Aveiro, pp.103-25.

Harris, H.H., Pickering, I.J., George, G.N. (2003). The chemical form of mercury in fish. *Science*, 301: 1203.

INE (2011). Instituto Nacional de Estatística. Censos 2011: resultados provisórios.

Jorge, I. and Sobral, M. P. (2004). Alguns aspectos da biologia e da ecologia do choco, *Sepia officinalis* (Linnaeus, 1758), da região de Aveiro. *Relatório Técnico-Científico do IPIMAR, Série digital* (http://ipimar-iniap.ipimar.pt), nº15, 29 p.

Lacoue-Labarthe, T., Warnau, M., Oberhänsli, F. Teyssié, J.L., Bustamante, P. (2009). Bioaccumulation of inorganic Hg by the juvenile cuttlefish *Sepia officinalis* exposed to ^{203}Hg radiolabelled seawater and food. *Aquatic Biology*, 6:91-98.

Lee, C. L., Fang, M. D., Hsieh, M. T. (1998). Characterization and distribution of metals in superficial sediments in Southwestern Taiwan. *Marine Pollution Bulletin*, 36: 464-471.

Leistel, J.M., Marcoux, E., Thiéblemont, D., Quesada, C., Sánchez, A., Almodovar, G.R., Pascual, E., Sáez R. (1998). The volcanic-hosted massive sulfide deposits of the Iberian Pyrite Belt. Review and preface of the thematic issue. *Miner Deposit*, 33, 2-30.

Lima, C. (1986). Impacto da poluição por mercúrio nos organismos aquáticos da Ria de Aveiro. *Relatório Instituto Nacional de Investigação das Pescas*, 66: 1-28.

Lopes, J.F., Dias, J.M., Dekeyser, I. (2006). Numerical modelling of cohesive sediments transport in the Ria de Aveiro lagoo, Portugal. *Journal of Hydrology*, 319: 176-198.

Lopes, F.J., Dias, J.M. (2007). Residual circulation and sediment distribution in the Ria de Aveiro lagoon, Portugal. *Journal of Marine Systems*, 68:507-528.

Lucas, M.F., Caldeira, M.T., Hall, A., Duarte, A.C., Lima, C. (1986). Distribution of mercury in the sediments and fishes of the lagoon of Aveiro, Portugal. *Water Science Technology*, 18: 141-148.

Mangold, K. (1983). *Octopus vulgaris*. *In*: Boyle P. (Ed.), Cephalopod Life Cycles, Species Accounts, vol. I. *Academic Press*, United Kingdom, 335-364.

Martin, J. and Flegal, A. (1975). High copper concentrations in squid levers in association with elevated levels of silver, Cd, and Zn. *Marine Biology*, 30: 51-55.

May, T.W., Wiedmeyer, R.H., Brumbaugh, W.G., Schmitt, C.J. (1997). The determination of metals in sediment pore waters and in 1 N HCl extracted sediments by ICP-MS. *Atomic* Spectroscopy, 18 (5): 133-139.

Miramand P. and Guary, F.M. (1980). High concentrations of some heavy metals in tissues of the Mediterranean octopus. *Bulletin of Environmental Contamination and Toxicology*, 24: 783-788.

Miramand, P. and Bentley, D. (1992). Concentration and distribution of heavy metals in tissues of two cephalopods, *Eledone cirrhosa* and *Sepia officinalis*, from the French coast of the English Channel. *Marine Biology*, 114: 407-414.

Miramand, P., Bustamante, P., Bentley, D., Kouéta, D. (2006). Variation of heavy metal concentrations (Ag, Cd, Co, Cu, Fe, Pb, V, Zn) during the life cycle of the common cuttlefish *Sepia officinalis*. *Science of the Total Environment*, 361: 132-143.

Monteiro, L.R., Porteiro, F.M., Gonçalves, J.M. (1992). Inter- and intraspecific variation of mercury levels in muscle of cephalopods from the Azores. *Arquipélago Life Earth Sciences* 10: 13-22.

Monteiro, L.R., Costa, V., Furness, R.W., Santos, R.S. (1996). Mercury concentrations in prey fish indicate enhanced bioaccumulation in mesopelagic environments. *Marine Ecololy Progress Series*, 141: 21-5.

Monterroso, P., Abreu, S.N., Pereira, E., Vale, C., Duarte, A.C. (2003). Estimation of Cu, Cd, and Hg transported by plankton from a contaminated area (Ria de Aveiro). *Acta Oecologica*, 24: S351-S357.

Newell, R.C., Seiderer, L.J., Hitchcock, D R. (1998). The impact of dredging works in coastal waters: a review of the sensitivity to disturbance and subsequent recovery of biological resources on the sea bed. *Oceanography and Marine Biology*, 36: 127-178.

OSPAR Commission. (2004). Mercury losses from the Chlor-Alkali Industry (1982-2002).

Pastorinho, M.R., Telfer, T.C., Nogueira, A.J.A., Soares, A.M.V.M., Ranville, J.F. (2012). An evaluation of trace metal distribution, enrichment factos and risk in sediments of a coastal lagoon (Ria de Aveiro, Portugal). *Environmental Earth Science*, DOI: 10.1007/s12665-012-1643-xOnline First™.

Pato, P., Lopes, C., Válega, M., Lillebø, A.I., Dias, J.M., Pereira, E., Duarte, A.C. (2008) Mercury fluxes between an impacted coastal lagoon and the Atlantic Ocean. *Estuarine, Coastal and Shelf Science*, 76: 787-796.

Pereira, E., (1996). Distribuição, Reactividade e Transporte do mercúrio na ria de Aveiro. Ph.D. Thesis. Universidade de Aveiro, Departamento de Química, pp. 284.

Pereira, M.E., Duarte, A.C., Millward, G., Abreu, S.N., Vale, C. (1998a). An estimation of industrial mercury stored in sediments of a confined area of the lagoon of Aveiro (Portugal). *Water Science and Technology*, 37(6-7): 125-130.

Pereira, M.E., Duarte, A.C., Millward, G., Vale, C., Abreu, S.N. (1998b). Tidal export of particulate mercury from the most contaminated area of Aveiro´s Lagoon, Portugal. *Science of the Total Environment*, 213: 157-163.

Pereira, E., Abreu, S.N., Coelho, J.P., Lopes, C.B., Pardal, M.A., Vale, C., Duarte, A.C. (2006). Seasonal fluctuations of tissue mercury contents in the european shore crab *Carcinus maenas* from low and high contamination areas (Ria de Aveiro, Portugal) *Marine Pollution Bulletin*, 52: 1450-1457.

Pereira, M.E., Lillebø, A.I., Pato, P., Válega, J.P., Coelho, C.B., Lopes, C.B., Rodrigues, S., Cachada, A., Otero, M., Pardal, M.A., Duarte, A.C. (2009a). Mercury pollution in Ria de Aveiro (Portugal): a review of the system assessment. *Environmental Monitoring and Assessment*, 155(1-4): 39-49.

Pereira, P., Raimundo, J., Vale, C., Kadar, E. (2009b). Metal concentrations in digestive gland and mantle of Sepia officinalis from two coastal lagoons of Portugal. *Science of the total Environment*, 407: 1080-1088.

Pierce, G.J., Stowasser, G., Hastie, L.C., Bustamante, P. (2008). Geographical, seasonal and ontogenetic variation in cadmium and in mercury concentrations in squid (Cephalopoda: Teuthoidea) from UK waters. *Ecotoxicology and Environmental Safety*, 70: 422-432.

Pinczon du Sel, G., Blanc, A., Daguzan, J. (2000). The diet of the cuttlefish *Sepia Officinalis* L. (mollusc:cephalopoda)) during its life cycle in the Northern Bay of Biscay (France). *Aquatic Sciences*, 61:167-178.

Pombo, L. (2005). Diversity, population dynamics and fish production capacity in a coastal lagoon – the Ria de Aveiro. Ph.D. Thesis, University of Aveiro, pp.272.

Raimundo, J., Caetano, M., Vale, C. (2004). Geographical variation and partition of metals in tissues of *Octopus vulgaris* along the Portuguese coast. *Science of the Total Environment*, 325: 71-81.

Raimundo, J., Vale, C., Canário, J., Branco, V., Moura, I. (2010). Relations between mercury, methyl-mercury and selenium in tissues of *Octopus vulgaris* from the Portuguese Coast. *Environmental Pollution*, 158: 2094-2100.

Ramalhosa, E., Monterroso, P., Abreu, S., Pereira, E., Duarte, A. (2001). Storage and export of mercury from a contaminated Bay (Ria de Aveiro, Portugal). *Wetlands Ecology and Management*, 9: 311-316.

Ramalhosa, E., Pereira, E., Vale, C., Válega, M., Duarte, A.C. (2005). Distribution of mercury in the upper sediments from a polluted area (Ria de Aveiro, Portugal). *Marine Pollution Bulletin,* 50: 682-697.

Ramalhosa, E., Pato, P., Monterroso, P., Pereira, E., Vale, C., Duarte, A.C. (2006). Accumulation versus remobilization of H gin sediments of a contaminated lagoon. *Marine Pollution Bulletin*, 52(3): 353-356.

Reis, A.T., Rodrigues, S.M., Araújo, C., Coelho, J.P., Pereira, E., Duarte, A.C. (2009). Mercury contamination in the vicinity of a chlor-alkali plant and potential risks to local population. *Science of the Total Environment*, 407: 2689-2700.

Relvas, P., Barton, E.D., Dubert, J., Oliveira, P.B., Peliz, A., Silva, J.C.B, Santos, A.M.P. (2007). Physical oceanography of the western Iberia ecosystem: lastest views and challenges. Progress in *Oceanography,* 74 (2-3): 149-173.

Renzoni, A., Bacci, E., Falciai, L. (1973). Mercury concentration in the water, sediments and fauna of an area of the Tyrrhenian coast. *Revue Internationale d'Oceanographie Medicale,* 31-32: 17-45.

Rossi, A., Pellegrini, D., Belcari, P., Barghigiani, C. (1993). Mercury in *Eledone cirrhosa* from the Northern Tyrrhenian Sea: contents and relations with life cycle. *Marine Pollution Bulletin,* 26(12): 683-6.

Schuhmacher, M., Batiste, J., Bosque, M.A., Domingo, J.L., Corbella, J. (1994). Mercury concentrations in Marine species from the coastal area of Tarragona Province, spain. Dietary intake of mercury through fish and seafood consumption. *Science of the Total Environment*, 156 (3): 269-273.

Seixas, S., Bustamante, P., Pierce, G. (2005a). Accumulation of mercury in the tissues of the common octopus *Octopus vulgaris* (L.) in two localities on the Portuguese coast. *Science of the Total Environment*, 340:113-122.

Seixas, S., Bustamante, P., Pierce, G.J. (2005b). Interannual patterns of variation in concentrations of trace elements in arms of *Octopus vulgaris*. *Chemosphere*, 59: 1113-1124.

Smith, J.D., Plues, L., Heyraud, M., Cherry, R.D. (1984). Concentrations of the elements Ag, Al, Ca, Cd, Cu, Fe, Mg, Mn, Pb and Zn, and the radionuclides ^{210}Pb and ^{210}Po in the digestive gland of the squid *Notodarus gouldi*. *Marine Environmenta. Research*, 13: 55-68.

Sparks, T.H., Scott, W.A., Clarke, R.T. (1999). Traditional multivariate techniques: Potential for use in ecotoxicology. *Environmental Toxicology and Chemistry*, 18 (2): 128-137.

Stoeppler, M. and NÜrnberg, H.W. (1979). Comparative studies on trace metal levels in marine biota: III. Typical levels and accumulation of toxic trace metals in muscle tissue and organs of marine organisms from different European seas. *Ecotoxicology and environmental Safety*. 3(4): 335-351.

Storelli, M.M. and Marcotrigiano, G.O. (1999). Cadmium and total mercury in some cephalopods from the South Adriatic Sea (Italy). *Food Additives and Contaminants*, 16(6): 261-5. *In*: Bustamante P., Lahaye V., Durnez C., Churlaud C., Caurant F. (2006a). Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environmental*, 368: 585-596.

Van den Brink, P.J., Van den Brink, N.W., Ter Braak, J.F. (2003). Multivariate Analysis of Ecotoxicological data using ordination: demonstrations of utility on the basis of various examples. *Australasian Journal of Ecotoxicology*, 9: 141-156.

Valentim, J.M., Vaz, N., Silva, H., Duarte B., Caçador, I., Dias, J.M. (2013). Tagus estuary and Ria de Aveiro salt marsh dynamics and the impact of sea level rise. Estuarine, Coastal and Shelf Science, 1-14.

Warren, L.A., Tessier, A., Hare, L. (1998). Modelling cadmium accumulation by benthic invertebrates in situ: the relative contributions of sediment and overlying water reservoirs to organism cadmium concentrations. *Limnology and Oceanography*, 43 (7): 1442-1454.

CHAPTER V

Life-cycle and seasonal variation of metals accumulation (Fe, Zn, Cu, Cd, Pb and Hg) in common cuttlefish *Sepia officinalis*

V. Metals accumulation (Fe, Zn, Cu, Cd, Pb and Hg) during the life cycle of common cuttlefish *Sepia officinalis*

V.1. Abstract

The developmental changes in the concentration of 6 (essential and non-essential) metals (Fe, Zn, Cu, Cd, Pb and Hg) in two tissues (digestive gland and mantle) of the common cuttlefish *Sepia officinalis* caught in Ria de Aveiro were analysed from the stage I to Stage IV of maturation. No differences between sexes were identified in the four stages of maturity. After the immature individuals start to feed, the digestive gland appears to play a major role in the storage of all metals with the exception of Hg. Metal concentration of Zn and Cd in digestive gland increases with age during the life cycle of cuttlefish, Pb decreases from the stage I of maturity to the rest of the stages. Hg presented a positive correlation with age, however, unlike other metals, this correlation was observed in the mantle and not in the digestive gland, which suggests that the capacity of digestive gland to storage this metal is limited. However, digestive gland has a major role in Hg detoxification and depuration. The essential metals Fe and Cu displayed a homogeneous concentration of metal during growth of cuttlefish which revealed the regular balance of these metals. For the entire life cycle of cuttlefish the concentrations of essential metals were always higher than concentrations of non-essential metals. The results highlighted the strong capacity of cuttlefish to storage in its tissues essential and non-essential metals in different stages of its life cycle, suggesting that *Sepia officinalis* could be considered good specie for toxicological studies.

V.2. Introduction

The cuttlefish *Sepia officinalis* has a very short life cycle, during which migrations related to growth and reproduction take place (Miramand *et al.*, 2006). In Ria de Aveiro as in the English Channel and in other Atlantic inshore areas, the reproductive season, after which these organisms die, occurs between April and September of their second year of life, when they are aged between 14 and 18 months (Boyle, 1990; Boucaud-Carnou *et al.*, 1991; Legoff and Daguzan, 1991; Dias *et al*., in press). Consequently, the life of cuttlefish never exceeds 2 years in these areas (Richard, 1971; Boletzky, 1983). This extremely high growth rate of the cuttlefish is due to their high feeding rates, characteristic of cephalopods, moreover food has often been cited as the main uptake pathway of metal contamination in cephalopods (Martin and Flegal, 1975; Bustamante *et al.*, 1998b; Lacoue-Labarthe *et al.*, 2009).

Cuttlefish is initially benthopelagic but subsequently is mostly benthic, however this specie spend long periods buried into sediment (Nixon and Mangold, 1998). Sediment represents the main sink for pollutants in coastal ecosystems that are impacted by anthropogenic activities (Fichet *et al.*, 1999). Potentially toxic compounds, especially metals, are adsorbed on the mineral or organic particles, either in their organic or inorganic forms (Robbe, 1984 in Fichet *et al.*, 1999). Taking this into account, the contamination via sediment must be considered for the early stages of the cuttlefish life cycle, and the contamination via water in the embryogenic stage, since this stage occurs in direct contact with water and the animal only feeds from the reserves of the egg. As the principal via of contamination changes along the cuttlefish life cycle, it is expected that the patterns of metal accumulation also change during the growth and development of juveniles into adult cuttlefishes. In general, after the hatching, accumulation of toxic elements shows two patterns: metals such as Ag, which is accumulated immediately since juveniles, are in direct contact with seawater, and metals as Cd and Pb which are significantly incorporate only once the cephalopods start to feed (Miramand *et al.*, 2006).

Despite the short life cycle of *S. officinalis*, several studies have demonstrated the efficiency of cuttlefish as accumulators of various trace metals in their tissues, and also that the digestive gland acts as the principal organ in the bioaccumulation processes (Miramand and Bentley, 1992; Bustamante *et al.*, 1998a; Bustamante *et al*., 2002a,b), with the exception of Hg where the digestive gland has a major role in Hg detoxification and depuration, being limited it's capacity for storage this element (Lacoue-Labarthe *et al.*, 2009). Other authors studied the accumulation of metals in the early stages and during the growth of cuttlefish (Bustamante *et al.*, 2002b; Bustamante *et al.*, 2004; Bustamante *et al.*, 2006b; Miramand *et al.*, 2006; Lacoue-Labarthe *et al.*, 2009), however only one of these studies is from organisms captured in is natural environment (Miramand *et al.*, 2006). Overall, there is a lack of published data concerning the accumulation of essential and non-essential metals in juvenile cuttlefish captured in the wild and subject to the natural conditions of the environment. Such baseline information is needed to better understand the necessities and the processes of accumulation of trace metals along the cuttlefish life cycle. The main aim of this study is to understand the seasonal and spatial changes in metal concentrations during the growth, life cycle of *S. officinalis*, for those 6 metals (Fe, Cu, Zn, Cd, Pb and Hg) investigated from immature to adult cuttlefish, in Ria de Aveiro. This investigation focused on *S. officinalis*, due to its wide distribution in coastal waters and high commercial importance in Portugal.

V.3. Materials and methods

V.3.1. Study Area

Ria the Aveiro ia a shallow well-mixed (marine and freshwater) coastal lagoon on the northwester coast of Portugal (40°38´N, 8°44´W) connected to the sea by a single channel (1.3 Km long, 350 m wide and 20 m deep). (Dias and Fernandes, 2006; Abrantes *et al.*, 2006). The system can be subdivided into channels that, due to specific characteristics, can be regarded as independent estuaries connected to a common outlet. In general, the most common typology is one of extensive intertidal zones, namely mud flats and salt marshes, with the exception of the central part of the lagoon (Picado *et al.*, 2010; Pastorinho *et*

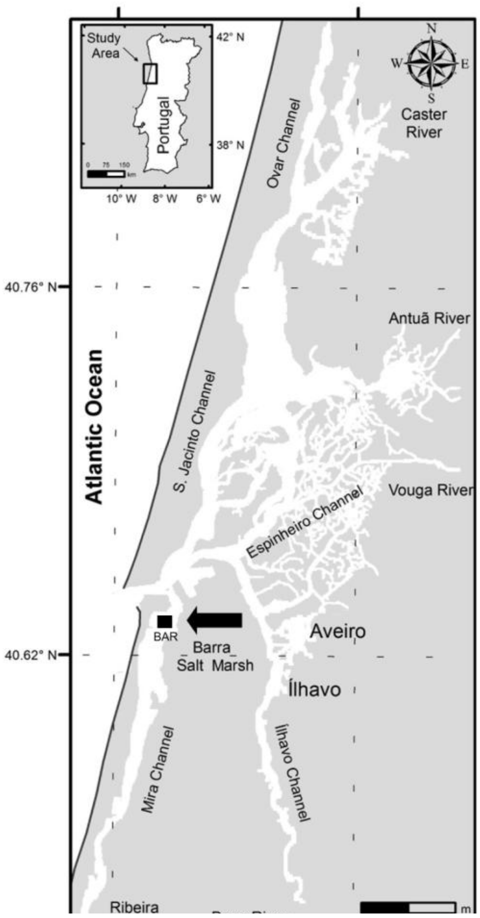

Figure V.1 - Sampling station of *S. officinalis* in *Ria de* Aveiro (Adapted from Valentim *et al*., 2013) *al*., 2012). The hydrodynamic pattern of the lagoon is imposed by tide, each channel possesses its own hydrodymanics influence by the respective tidal prism, amount of freshwater input and wind force. Strongest currents are observed at the inlet channel, reaching values higher than 2 m/s (Vaz *et al*., 2009). The bathymetry shows that Ria de Aveiro is a very shallow lagoon (average depth of 3 m). The deepest areas of the lagoon are confined to the inlet channel and to small areas close to the lagoon mouth, at its western boundary, where the depths may reach values of 20 m. In the navigation channels, that are frequently dredged, the depths are about 7 m (Lopes and Dias, 2007).

V.3.2. Sampling and biological data collection

A total of 68 cuttlefishes were analysed, and the concentrations of iron (Fe), zinc (Zn), cooper (Cu), cadmium (Cd), lead (Pb) and mercury (Hg) were determined. Cuttlefish, *Sepia officinalis*, was caught monthly during March 2003 to December 2003 with a network trammel and in two periods corresponding to spring and summer with a traditional beach-seine named “chincha”. The use of two types of gears was to ashore the capture of individuals of a wide range of sizes and consequently, individuals from immature stage (“chincha”) to adult stage (network trammel). Sampling was conducted in a single site of the Ria de Aveiro, Barra (BAR), corresponding to the initial part of the Mira channel, close to the entrance of the lagoon and subject to considerable vessel traffic (Figure V.1). The choice of the sampling site took into account several factors: site of easy and safe access for sampling; area subjected to regular dredging, which leads to large variations in the concentration of suspended sediments and consequently, increasing the availability of contaminants resuspended in water column; and also an area used by local fisherman to capture cuttlefish. Abiotic parameters (water temperature (ºC); dissolved oxygen (mg/L); turbidity (m); pH; salinity) were assessed according APHA (1998) guidelines (Table V-I). For all the cuttlefishes, dorsal mantle length (DML) (mm), wet weight (WWt) (g), sex, and maturity stage were recorded (Table V-II). A standard 5-point scale of sexual maturity for *S. officinalis* was used (adapted from Alonso-Allende and Guerra, 1984), in which stage I is “immature”, stages II-III are ”maturing”, and stages IV-V are “mature”. Specimens with no visible gonad development were classified as “juveniles” (Challier *et al.*, 2002) and allocated to maturity stage I (Table V-II).Specimens were stored in individual plastic bags and immediately frozen, in order to minimize mobilization of metals between organs

(Martin and Flegal, 1975). In the laboratory, the digestive gland of each specimens was totally removed under defrost conditions without rupture of outer membrane. Mantle free from skin and inner membrane was also sampled. Tissues samples were weighted, frozen at – 80 °C and homogenized.

Table V-I – Abiotic parameters (average±SD) of the water from the Ria de Aveiro sampling season.

Season	Depth (m)	Turbidity (m)	Temperature (Cº)	Salinity	pH	Dissolved oxygen (mg/L)
Spring	2.9±2.1	0.80±0.64	16.5±1.42	34.6±1.4	8.19±0.19	8.98±0.93
Summer	2.6±0.9	0.71±0.54	19.4±1.04	35.2±1.5	8.10±0.92	9.07±1.34

Table V-II – Size, sex (F (♀) and M (♂)) and maturity stage in sampled *S. officinalis*, for each sampling season.

Season	n	Sex		DML	Maturity	Proporcion of mature
		F	M	(mm)		cuttlefish (%)
Spring	18	5	13	114±37	I-IV (III)	39
Summer	50	24	26	61±25	I-IV (I)	6

For dorsal mantle length (DML), the table shows the average±standard deviation. For maturity, the range and median values for the maturity stage index are presented. The proportion of mature cuttlefishes refers to stage IV-V.

V.3.3. Analysis of metals

Tissue samples were lyophilised and homogenised. Approximately 200 mg of the dry tissue was digested with a mixture of HNO_3 (sp, 65% v/v) and H_2O_2 (sp, 30% v/v) at different temperatures according to the method described in Ferreira *et al*. (1990). All laboratory ware was cleaned with HNO_3 (20%) for 2 days and rinsed with Milli-Q water to avoid contamination. The selection of the analytical methodology and apparatus to use in metal quantification was determined by metal concentration present in the tissues. Quantification of Fe, Zn and Cu in mantle and digestive gland and Cd in digestive gland was performed by flame atomic absorption spectrometry (Perkin Elmer Analyst 100) while quantification of Cd in mantle and Pb in the mantle and digestive gland were analysed with ICP-MS. Total Hg was determined by atomic absorption spectrometry, using a silicon UV diode detector Leco AMA-254 after pyrolysis of each sample in a combustion tube at 750 ºC, under an oxygen atmosphere, and collection on a gold amalgamator (Costley *et al.*, 2000). The accuracy of the analytical methods was assessed by the analysis of international certificate standards: DORM-1, DORM-2 (dogfish muscle); TORT-1, TORT-2 (lobster hepatopancreas) and DOLT-3 (fish liver) (National Research Council of Canada). The obtained values and certificated values do not differ significantly at 95% confidence level (Table V-III). Blanks and standard references materials were run together with samples. All the results are given in micrograms per gram of dry weight tissue (µg g^{-1} DWT). Under our analysis conditions, the detection limits (µg g^{-1}) were: 4.2 (Fe); 0.50 (Zn), 1.2 (Cu), 0.0055 (Cd), 0.0040 (Pb) and 0.041 (Hg). Precision errors varied from 1 to 8%.

Table V-III - Concentration of metals in certified reference materials used to accuracy analytical quality control (mean±SD; µg g^{-1} DWt).

Standard tissue		Fe (µg g^{-1})	Zn (µg g^{-1})	Cu (µg g^{-1})	Cd (µg g^{-1})	Pb (µg g^{-1})	Hg (µg g^{-1})
DORM-1	Present study	61.7±2.8	20.4±1.7	5.95±0.48	0.092±0.009	0.30±0.12	0.080±0.031
	Certified	63.5±5.3	21.3±1.0	5.22±0.33	0.086±0.012	0.40±0.12	0.080±0.074
DORM-2	Present study	-	24.0±2.0	-	0.041±0.002	0.05±0.01	-
	Certified	-	25.6±2.3	-	0.043±0.008	0.07±0.01	-
TORT-1	Present study	186.0±12.0	-	-	25.0±0.6	9.10±0.60	0.32±0.01
	Certified	186.0±11.0	-	-	26.3±2.1	10.40±2.00	0.33±0.06
TORT-2	Present study	93.5±2.1	177.0±0.8	98±2	-	0.51±0.16	0.29±0.006
	Certified	105.0±13.0	180.0±6.0	106±10	-	0.35±0.13	0.27±0.06
DOLT-3	Present study	-	-	-	-	-	3.24±0.04
	Certified	-	-	-	-	-	3.37±0.14

V.3.4. Statistical analysis

Specific weight metal concentrations were calculated as a function of the dry weight. Normality of data and homogeneity of variance were tested using the Anderson-Darling test and Levene´s test, respectively. Whenever significant deviations from normality and homoscedasticity were found data was log-transformed prior to further statistical analysis to correct for these deviations and normalize the data. Whenever significant differences were found, between sex and maturity stage, a two-way ANOVA followed by post hoc Tukey comparisons were performed for each metal. Pairing season was also used to assess seasonality effects using a two way ANOVA. Statistical testing was carried out with MINITAB® Release 14.20. A significance level of 0.05 was used for all tests.

V.4. Results

V.4.1. Relationship between mantle length (DML) and wet weight (WWt)

Through the use of two fishing arts for the capture of cuttlefish it was possible to obtain the various stages of maturation (I-IV) of the life cycle of *S. officinalis.* The analysed data included specimens from different sizes and weights over wide ranges, and also included individuals of both sex (M (♂) and F (♀)) (Table V-II). The relationships between size and weight of all analysed cuttlefishes presented a typically allometric curve (Figure V.2).

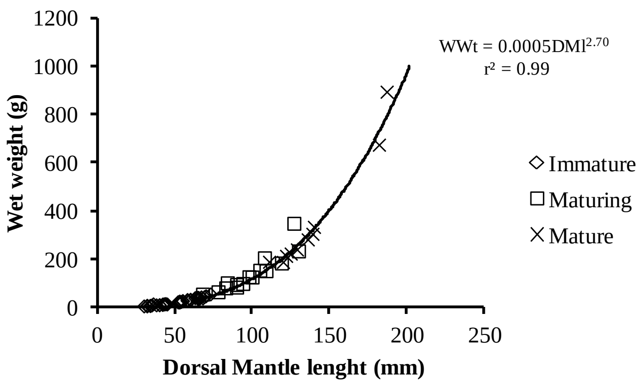

Figure V.2 - Allometric relation between total wet weight (g) and mantle length (mm) of the *S. officinalis* captured in the Ria de Aveiro, making reference to de maturity stage of each specimen (stage I – Immature; stage II and III – Maturing and stage IV – Mature).

Weight and length present a strong correlation, with proportional increase over growth. The correlation was very significant ($p<0.001$) and Pearson correlation of 0.995. There is not a clear cut-off point marking the transition between maturity stages (Figure V.2), instead some overlap exists in particular between the maturing and mature stages.

V.4.2. Metal concentrations and distribution among tissues

Metal concentrations in mantle of *S. officinalis* belonging to different stages of maturity (stages I, II, II and IV) from Barra in Ria de Aveiro are given in Table V-IV. The highest metal concentrations correspond to those of the essential metals Cu, Fe and Zn (between 9 and 63 µg g^{-1} DWt) and the lowest to non-essential metals (between 0.05 and 1.35 µg g^{-1} DWt). For mantle there was no significant differences ($p<0.05$; $F_{1, 65}=2.22$) associated with sex, thus for the rest of the analysis the individuals were considered all together. Highly significant ($p<0.001$) differences between seasons (spring and summer) were detected for Zn and Hg concentrations. Levels of Zn and Hg were higher in animals captured during the spring season (Table V-V).

Table V-IV – DML: Dorsal mantle length (average±SD; mm), n: number of individuals analysed, Metal concentrations (average±SD; µg g^{-1} DWt) in mantle and digestive gland of *Sepia officinalis* sampled in Ria de Aveiro at different stages of its life cycle.

	DML (mm)	n	Fe		Zn		Cu		Cd		Pb		Hg	
			Mantle#	DG#	Mantle#	DG#	Mantle#	DG#	Mantle*	DG#	Mantle*	DG*	Mantle°	DG°
Stage I	51.0 ± 1.2	43	26 ± 20	622 ± 293	56 ± 9	243 ± 112	14 ± 8	301 ± 169	1.35 ± 3.09	5.3 ± 3.9	0.07 ± 0.04	2.40 ± 1.84	0.18 ± 0.05	0.46 ± 0.17
Stage II	89.0 ± 1.4	8	20 ± 8	424 ± 150	57 ± 13	578 ± 270	9 ± 3	446 ± 236	0.32 ± 0.26	8.8 ± 10.7	0.09 ± 0.08	0.92 ± 0.26	0.27 ± 0.10	0.51 ± 0.15
Stage III	110.0 ± 1.5	7	22 ± 9	596 ± 168	63 ± 7	996 ± 396	11 ± 4	264 ± 214	1.54 ± 3.30	33.1 ± 24.9	0.05 ± 0.08	1.25 ± 0.35	0.35 ± 0.07	0.53 ± 0.06
Stage IV	139.0 ± 1.6	10	20 ± 11	555 ± 163	63 ± 5	782 ± 224	9 ± 3	334 ± 486	0.49 ± 0.52	78.0 ± 17.7	0.41 ± 1.18	1.62 ± 1.45	0.36 ± 0.04	0.68 ± 0.52

measured with Flame Atomic Spectometer   * measured with ICP-MS   ° measured with atomic absorption spectrometry

The tissue type (mantle and digestive gland) had a highly significant ($p<0.001$) influence on the accumulation of Fe, Zn, Cu, Cd and Pb being one or two orders of magnitude higher than in mantle (Table V-IV). Like for mantle, in digestive gland there was no significant difference ($p<0.05$) between metal accumulation with sex. Only Zn and Cd concentrations presented significant differences ($p<0.001$) between seasons (spring and summer). For both metals the accumulation was higher in spring than in summer (Table V-V).

V.4.3. **Variation of metal concentrations along life cycle of *S. officinalis***

Metal concentrations in digestive gland, principal organ of storage, showed significant variations throughout the life cycle of cuttlefish (Table V-V). As expected the concentrations of the essential metals (Fe, Zn, and Cu) were much higher than the non-essential metals (Cd, Pb and Hg) and the mean values of metals analysed, for digestive gland, were in the following order: Zn>Fe>Cu>>Cd>Pb>Hg (Table V-IV and Table V-V). This order is always verified in all maturity stages with the exception of stage I (immature specimens) were the mean values of Fe and Cu concentrations were higher than mean value of Zn concentration (Table V-IV).

Metal concentrations of Fe and Cu did not differ throughout the different stages of the life cycle of the cuttlefish, for both analysed tissues (Table V-V). Zinc (Zn) presented the highest concentrations of both tissues and increased notably from stage I to other stages II, III and IV, this increment was significant ($p<0.001$), (Table V-V and Figure V.3).

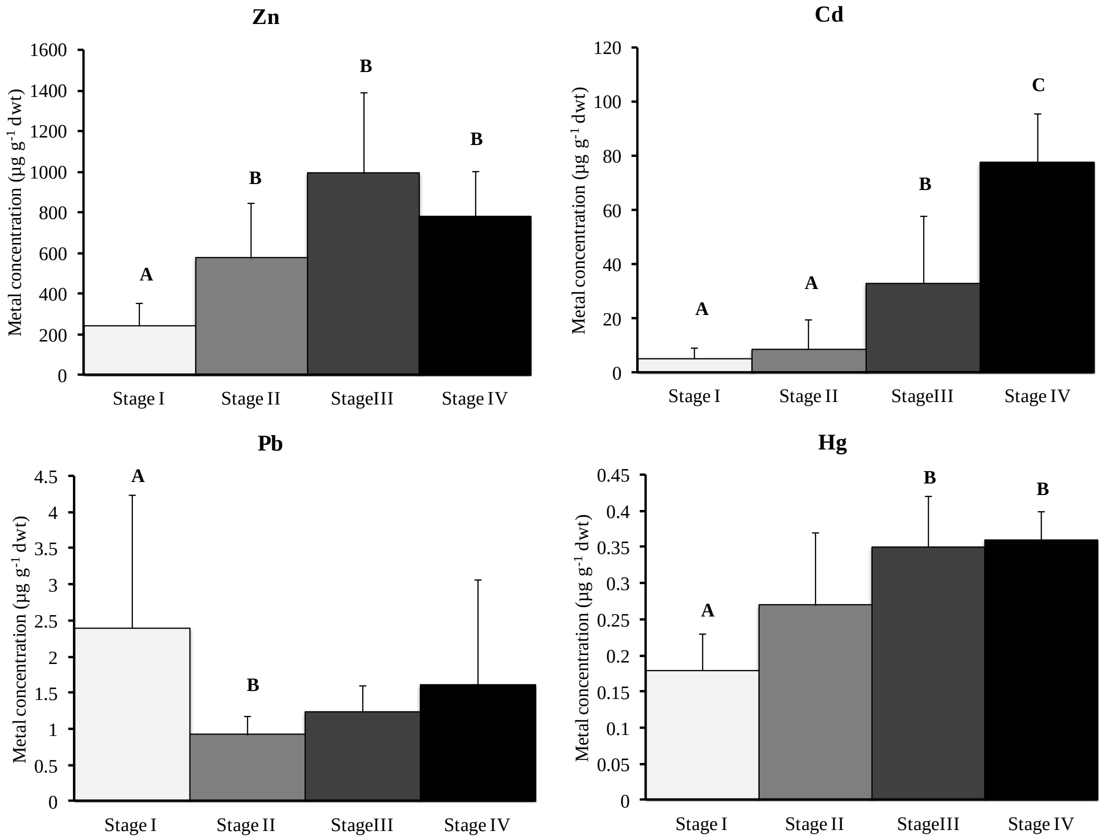

Figure V.3 - Comparison of the mean values (µg g^{-1}.dry weight) and the standard deviation in the digestive gland of Zn, Cd, Pb and Hg among life cycle of *S. officinalis*. Bars with the same letters were not significantly different ($p>0.05$) and bars with no letter were not conclusive.

This metal showed a positive correlation with mantle length (age) ($p<0.001$, $F_{1,66}=14.19$), so Zn increased significantly in a allometric way (Figure V.4). For cadmium was observed a substantial increase when comparing the immature individuals with the mature individuals, in the digestive gland. The accumulation was similar in the two first stages (stages I and II) of maturity but different in later stages (stages III and IV) (Table V-V). For Pb, in terms of tissue type (mantle and digestive gland) a very high value (three orders of magnitude higher than in mantle) was obtained in the stage I for digestive gland, while for mantle the higher value belongs to stage IV (two orders of magnitude higher than other stages in mantle). Pb tended to decrease significantly from stage I to stage II and then increase for stage III and IV (Table V-V and Figure V.3) ($p<0.001$, $F_{1,66}=41.24$; $p<0.001$, $F_{1,66}=21.62$, respectively). Hg showed the same increase pattern between the stages of maturity in both tissues, however only for mantle this differences were significant ($p<0.001$, $F_{3,\ 64}=24.10$). (Table V-V). Among toxic metals, Hg was the only metal showing a linear significant increase with age ($F_{1,\ 66}=14.19$; $p<0.001$), (Figure V.4).

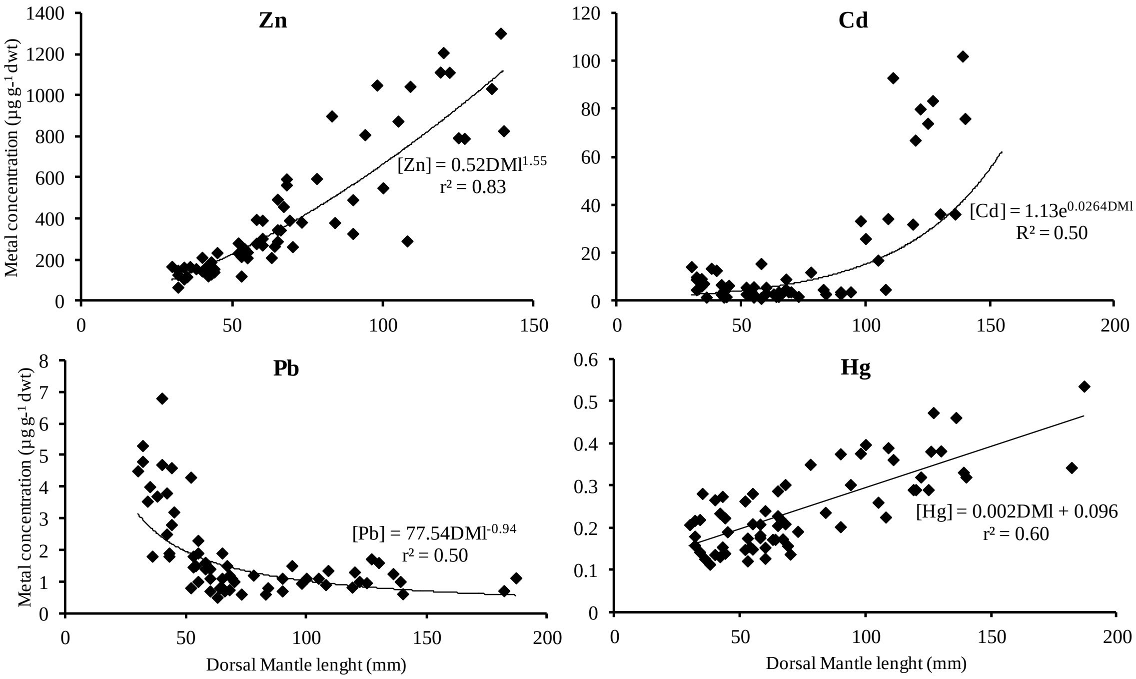

Figure V.4 – Variations of metal concentrations (µg g^{-1} DWt) in the digestive gland for Zn, Cd and Pb and in mantle for Hg with the dorsal mantle length (mm), for the specie, *Sepia officinalis*.

Table V-V - Factorial analysis of variance (ANOVA) testing statistical comparisons between seasons and maturity stage for mantle and digestive gland of *S. officinalis* on the concentration of essential metals (Fe, Zn and Cu) and non-essential metals (Cd, Pb and Hg).

		Mantle			**Digestive Gland**		
Metals	Factors	F	Significance (p value)	Conclusions	F	Significance (p value)	Conclusions
Fe	Season	$F_{1, 66}$=0.25	P=0.62	NS	$F_{1, 66}$=0.00	P=0.94	NS
	Maturity stage	$F_{3, 64}$=0.66	P=0.58	NS	$F_{3, 64}$=2.20	P=0.097	NS
Zn	Season	$F_{1, 66}$=25.07	<0.001 (S)	spring≠summer	$F_{1, 66}$=55.23	<0.001 (S)	spring≠summer
	Maturity stage	$F_{3, 64}$=32.78	0.019 (S)	I≠IV	$F_{3, 64}$=19.18	<0.001 (S)	I≠II=IV=III
Cu	Season	$F_{1, 66}$=0.32	P=0.57	NS	$F_{1, 66}$=3.65	P=0.61	NS
	Maturity stage	$F_{3, 63}$=1.17	P=0.33	NS	$F_{3, 64}$=2.14	P=0.104	NS
Cd	Season	$F_{1, 66}$=1,49	P=0.23	NS	$F_{1, 66}$=36.99	<0.001 (S)	spring≠summer
	Maturity stage	$F_{3, 64}$=0.06	P=0.98	NS	$F_{1, 46}$=7.37	0.009 (S)	I=II≠III≠IV
Pb	Season	$F_{1, 65}$=1.66	P=0.203	NS	$F_{1, 66}$=3.95	P=0.051	NS
	Maturity stage	$F_{3, 64}$=2.68	P=0.054	NS	$F_{3, 64}$=3.13	0.032 (S)	II≠I
Hg	Season	$F_{1, 66}$=3.58	<0.001 (S)	spring≠summer	$F_{1, 66}$=0.19	P=0.67	NS
	Maturity stage	$F_{3, 64}$=24.10	<0.001 (S)	I≠III=IV	$F_{3, 64}$=2.04	P=0.12	NS

V.5. Discussion

The strong correlation between length and weight is principally due to environment. Cuttlefishes in the different stages of their life cycle are subjected to different conditions in the environment, since they are migratory species, and as we known changes in the abiotic parameters can interfere with the process of maturation of the individual.

Most of the studies that focus on the metal bioaccumulation in cephalopods are related to a single organ, mainly the digestive gland known to play a major role in the energetic metabolism of cephalopods. These works although limited to a restrict number of cephalopod species have highlighted that this organ is deeply involved in the metabolism of Zn, Cu and Cd (Martin and Flegal, 1975; Smith *et al.*, 1984; Finger and Smith, 1987; Miramand and Guary, 1980; Bustamante *et al.*, 1998b, 2002a). Metal concentrations in mantle and digestive gland of *S. officinalis* along several maturity stages (Stage I, II, III and IV) analysed showed distinct levels of bioaccumulation for both tissues.

Indeed, the mean concentrations of Fe, Zn, Cu, Cd and Pb in the digestive gland are much higher than the mean concentrations of those metals in mantle. In contrast, for Hg the concentrations obtained for the digestive gland were higher than in mantle but of the same order of magnitude. Consequently, and in accordance to Miramand *et al.*, 2006, the digestive gland generally contained a great proportion of the whole-body burden of the metals with the exception of Hg (Bustamante *et al.*, 2006a).

Several authors refer that mantle is a tissue with strong capacity for storage large amounts of Hg (Renzoni *et al.,* 1973; Monteiro *et al.,* 1992; Seixas *et al.,* 2005). Such a different pattern of accumulation between Fe, Zn, Cu, Cd and Pb analysed and Hg suggest that cuttlefish has different mechanisms of uptake or sequestration of these metals (Pierce *et al*., 2008). This difference was also observed for other authors like: Raimundo *et al.* (2004); Seixas *et al.* (2005); Bustamante *et al.* (2006a). This may be due to an excretion function of Hg associated with the digestive gland (Lacoue-Labarthe *et al.,* 2009), and a preferential redistribution of Hg to muscular tissues as methylmercury (MeHg) (Bustamante *et al.*, 2006a).

The embryonic development of nautiluses, cuttlefishes, squids and cirrate octopods occur in an eggs protected by a capsule whose thickness varies according to the species (Villanueva and Bustamante, 2006). Inside the egg of cuttlefish, concentrations of non-essential elements, as Ag, Cd and Pb, remains very low, which suggest a restrict transfer of these metals through the eggshell during the embryonic development (Miramand and Bentley, 1992; Bustamante *et al.*, 2002b, 2004; Miramand *et al.,* 2006). Moreover, eggs of cirrate octopods (en ex., *O. vulgaris*) lacks eggshell and the chorion is in direct contact with seawater. Taking this into account *O. vulgaris* eggs have higher concentrations for most of the essential elements and also for some of the non-essentials (i.e., Ag and Pb). This difference with encased eggs could be due to the absorption of these elements from the seawater during the embryonic development in *O. vulgaris* (Villanueva and Bustamante, 2006). Collected data are in agreement with these facts, indeed, Zn, Cd and Hg presented higher concentrations in mature stages than in immature stages. However, the most striking result was the extremely high value of Pb in the digestive gland of immature specimens. This aspect can be related with the source of intake of this metal. To the best of our knowledge, no data on the respective proportions of elements incorporated from food and seawater has been published for cephalopods. So, two possibilities were considered for this value, the food ingested by immature specimens was probably highly contaminated by Pb or the specimens were subjected to an acute exposition and recent uptake of Pb by seawater. On these immature specimens, being on an early stage of maturation, the digestive gland may not be working properly, leading only to the accumulation of metal and not to its detoxification. The decrease observed for the second stage of maturity reinforces this idea (Figure V.3). Further investigations on the uptake of Pb are needed to assess this hypothesis. It was shown (Miramand *et al.* 2006) that embryos present very low metal concentrations compared to older cuttlefish, and that concentrations of embryos were close or even identical to those measured in the vitellus of newly spawned eggs, which suggests that the vitellus contains a sufficient amount of essential metals (Cu, Fe, and Zn) needed for the development of the embryos. Considering the classification for cuttlefish age (Miramand *et al.* 2006), the immature cuttlefishes here analysed have 1 to 2 months, and so have already started to feed. Food is the major source of essential elements such as Cu, Fe and Zn (Bustamante, 1998; Bustamante *et al.*, 2002b), which have low concentrations in seawater, and digestive gland acts in the regulation of Fe, Zn and Cu. Despite dietary intakes, the concentrations of Fe and Cu in analysed tissues did not vary significantly among cuttlefish life cycle (Table V-IV) and only Zn showed a significant increase of the concentration in the analysed tissues (Figure V.3). This could be due to mechanisms of homeostatic regulation (Miramand *et al.*, 2006).

In digestive gland, Zn presented a significant increase ($p<0.05$) from stage I (243±112 µg g^{-1}.DWT) to stage II (578±270 µg g^{-1}.DWT) (Figure V.3), stage I are increased 2.4 times compared to stage II. This increase between immature and juveniles could be due to differences in efficiency of their digestive metabolism (Mangold, 1989 in Bustamante *et al*., 2002b). Regardless of being non-significant ($p>0.05$) Zn concentration in digestive gland almost doubled from stage II (immature adults) to stage III (pre-posture), this fact might be related to metal physiological changes during sexual maturation as reported for *S. officinalis* from the English Channel (Miramand *et al.*, 2006). In contrast, the decrease of Zn from stage III (pre-posture) to stage IV (posture) may result from higher metabolic requirements in immature individuals to reach maturation (Bustamante *et al.*, 2002a,b).

The digestive gland plays a major role in the energetic metabolism of cephalopods (Boucaud-Camou and Boucher-Rodoni, 1983 *in* Bustamante *et al.*, 2006a) and is known for accumulating metal in large amounts (Martin and Flegal, 1975; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante *et al.*, 2000), especially for Cd (Figure V.4 and Figure V.3). Indeed, during the life cycle of cuttlefish, specifically between stage I and stage IV, Cd concentrations increased 14 times compared to a factor of 2 for mantle. Cd is strongly retained in the tissue of adult cuttlefishes and this increased retention capacity occurs almost exclusively in the digestive gland. Several authors reported that the digestive gland play a primary role in the accumulation of Cd in cuttlefish (Miramand and Bentley, 1992; Bustamante, 1998; Bustamante *et al.*, 2002a). Thus, assimilated Cd that is contained in the digestive gland may be considered as actually stored. The lower retention efficiency for Cd noted in immature cuttlefish would result from the incomplete development of the digestive gland in early juveniles (Bustamante *et al.,* 2002b) and a restrict transfer of this non-essential metal from food in earlier stages. Cephalopods such as *Sepia officinalis* accumulate Cd mainly from diet (Bustamante *et al.*, 2002a,b).

Cephalopods are carnivorous, active predators and because they have very high feedings rates, most part of the metals can be assumed to be incorporated via food (Villanueva and Bustamante, 2006). Intake pathways also take place from seawater, as it occurs for Ag (Bustamante *et al.,* 2004, Miramand *et al.*, 2006). However, for all metals (including Hg) there are three possible sources of intake: water (Bustamante *et al.*, 2006a), food (Lacoue-Labarthe *et al.*, 2009) and sediments (Miramand *et al.*, 2006). Nonetheless, it has been reported that Hg is mainly accumulated from food, contributing to almost 80% of the global metal bioaccumulation (Lacoue-Labarthe *et al.*, 2009). Mercury is known for is capacity of bioaccumulate in marine organisms (Baeyers *et al.,* 2003; Bustamante *et al.*, 2006a). The results obtained in this work highlighted this capacity, in fact Hg concentration increased significantly (Table V-V and Figure V.3) along the life cycle of cuttlefish, presenting an increment of 2 times between stage I and Stage IV.

V.6. Conclusions

Comparison of metal concentrations in different maturity stages of cuttlefish show that cephalopods may accumulate differently along life cycle depending on physiological changes and also the characteristics of the analysed element. The results allowed the determination of three distinct groups of metals: the ones where the accumulated value decreased along the life cycle, as Pb; those where the accumulation increased in the tissues with growth (Zn, Cd and Hg); and finally, the metals that remained constant along growth, like Fe and Cu. Essential metal (Fe, Zn and Cu) always presented higher values than non-essential metals (Cd, Pb and Hg) regardless of the maturity stage of the specimen. The main stages of its life are punctuated by important seasonal migrations between shallow waters in summer and deeper waters in winter (Gauvrit *et al.*, 1997). These migrations may modify the bioaccumulation processes of metals, whereas during the migrations the animals move from coastal areas (with metallic inputs of anthropogenic origin) to offshore (presumably less polluted than the coast) (Miramand *et al.*, 2006). For a better understanding of the accumulation process of metals during the life cycle of cuttlefish, further research focusing these migrations are needed to improve our knowledge. Indeed, it would be very important to capture cuttlefish before the entrance in the Ria de Aveiro to obtain metal concentrations without anthropogenic pollution sources that exist within the lagoon.

V.7. References

Abrantes, I., Dias, J., Rocha, F. (2006). Spatial and temporal variability of suspended sediments concentration in Ria de aveiro and fluxes between the lagoon and the ocean. *Journal of Coastal Research*, 39: 718-723.

Alonso-Alende, J.M. and Guerra, A. (1984). Crustáceos decápodeos y moluscos de la ria de Vigo (mimeo). Presentado alos prémios de investigación 1984, Excma. Diputación de Pontevedra, 152 p.

APHA, (1998). *In*: Clesceri L.S., Greenberg A.E., Eaton, A.D. (Eds.). Standard Methods for the Examination of water and waste-water. *American Public Health Association*, Washington.

Baeyers, W., Leermakers, M., Pappina, T., Saprykin, A., Brion, N., Noyen, J. (2003). Bioconcentration and biomagnifications of mercury and methylmercury in the North Sea and Scheldt estuary fish. *Archives of the Environmental Contamination and Toxicology*, 45: 157-163.

Barrosa, J.O. (1980). A Ria de Aveiro e a sua influência na economia da região. *Recursos Hídricos*, 1 (3): 83-96.

Boletzky, S.V., (1983).*Sepia officinalis*. *In*: Boyle, P.R. (Ed.), Cephalopod Life Cycles, vol.1. *Academic Press*, London, pp.31-52.

Borrego C., Cruz M.M., Gonçalves P. (1994). Estudo de avaliação da vulnerabilidade da capacidade de recepção das águas costeiras em Portugal. Relatório R 3.2. Meios receptores e suas características: (Be 1) – Ria de Aveiro, Pateira de Fermentelos e Barrinha de Mira. Projecto nº 13/94. IDAD, Aveiro.

Boucaud-Camou, E. and Boucher-Rodoni, R. (1983). Feeding and digestion in Cephalopods. In: Saleudin A.S.M., Wilbur K.M., editors. The Mollusca, vol. 5, Part.2 New York: *Academic Press*: p. 149-87. *In*: Bustamante P., Lahaye V., Durnez C., Churlaud C., Caurant F. (2006a). Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environmental*, 368: 585-596.

Boyle, P.R. (1990). Cephalopod biology in the fisheries context. *Fisheries Research*, 8(4): 303-321.

Bustamante, P. (1998). Etude des processus de bioaccumulation et de détoxification d´éléments traces (métaus lourds et terres rares) chez les mollusques cèphalopodes et bivalves pectinidés. Implication de leur biodisponibilité pour le transfert vers les prédateurs. Thesis, University of La Rochelle: pp.290.

Bustamante P., Caurant F., Fowler S., Miramand P. (1998a) Cephalopods as a vector for the transfer of Cd, to top marine predators in the North-East Atlantic Ocean. *Science of the Total Environment*, 220: 71-80.

Bustamante, P., Cherel Y., Caurant F., Miramand P. (1998b) Cadmiun, copper and Zinc in octopuses from Kerguelen Islands, Southern Indian Ocean. *Polar Biology*, 19: 264-271.

Bustamante, P., Grigioni S., Boucher-Rodoni R, Caurant F., Miramand P. (2000). Bioaccumulation of 12 trace elements in the tissues of the nautilus *Nautilus macromphalus* from New Caledonia. *Marine Pollution Bulletin*; 40: 688-96.

Bustamante, P., Cosson, R.P., Gallien I., Caurant, F., Miramand, P. (2002a). Cadmium detoxification processes in the digestive gland of cephalopods in relation to accumulated Cd concentrations. *Marine Environmental Research*, 53: 227-241.

Bustamante, P., Teyssié, J., Fowler, S.W., Cotret, O., Danis, B., Miramand, P., Warnau, M. (2002b). Biokinetics of zinc and cadmium accumulation and depuration at different stages in the life cycle of the cuttlefish *Sepia officinalis. Marine Ecology Progress Series*, 231: 167-177.

Bustamante, P., Teyssié, J.-L., Danis, B., Fowler, S.W., Miramand, P., Cotret, O., Warnau, M. (2004). Uptake, transfer and distribution of silver rand cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. *Marine Ecology Progress Series*, 269: 185-195.

Bustamante, P., Lahaye, V., Durnez, C., Churlaud, C., Caurant, F. (2006a). Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environment*, 368: 585-596.

Bustamante, P., Teyssié, J.L., Fowler, S.W., Warnau, M.. (2006b). Assessment of the exposure pathway in the uptake and distribution of americium and cesium in cuttlefish (*Sepia officinalis*) at different stages of its life cycle. *Journal of Experimental Marine Biology and Ecology*, 331: 198-207.

Challier, L., Royer, J., Robin, J-P. (2002). Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. *Aquatic Living Resources*, 15: 303-311.

Dias, J.M. (2001). Contribution to the study of the Ria de Aveiro hydrodynamics, PhD thesis, Universidade de Aveiro, Portugal, pp.288.

Dias, J.M., Lopes, J.F., Dekeyser, I. (1999). Hydrological characterization of Ria de Aveiro laggon, Portugal, in early summer. *Oceanologica Acta*, 22: 437-485.

Dias, J. M. and Fernandes, E.H. (2006). Tidal and subtidal propagation in two Atlantic Estuaries: Patos Laggon (Brazil) and Ria de Aveiro Lagoon (Portugal). *Journal of Coastal Research*, 39: 1422-1426.

Doca Pesca of Aveiro. (2013). Total landings of cuttlefish in Aveiro Lagoon by tones captured and annual turnover per year, since 2002 to 2012. Statistical Department.

Fichet, D., Boucher, G., Radenac, G., Miramand, P. (1999). Concentration and mobilization of Cd, Cu, Pb and Zn by meiofauna populations living in harbor sediment: their role in the heavy metal flux from sediment to food web. *Science of the Total Environment*, 243/244: 263-272.

Finger, J.M and Smith, J.D. (1987). Molecular association of Cu, Zn, Cd and ^{210}Po in digestive gland of the squid *Nototodarus gouldi*. *Marine Biology*, 95: 87-91.

Gauvrit, E., Le Goff R., Daguzan J. (1997). Reproductive cycle of the cuttlefish, *Sepia officinalis* (L.) in the Northern part of the Bay of Biscay. *Journal of Molluscan Studies*, 63: 19-28.

Hall, A., Lucas, M.F., Caldeira, M.T., Duarte, A.C. (1985). Distribuição de Mercúrio nos Sedimentos da Ria de Aveiro (Distribution of Mercury in the sediments of Ria de Aveiro, Portugal). Actas das jornadas da Ria de Aveiro. Poluição da Ria de Aveiro, I. Câmara de Aveiro, pp.103-25.

Lacoue-Labarthe, T., Warnau, M., Oberhänsli, F. Teyssié, J.L., Bustamante, P. (2009). Bioaccumulation of inorganic Hg by the juvenile cuttlefish *Sepia officinalis* exposed to ^{203}Hg radiolabelled seawater and food. *Aquatic Biology*, 6: 91-98.

Legoff, R.and Daguzan, J. (1991). Etude des déplacements de la seiche commune *Sepia officinalis* L. dans le Golfe du Morbihan au cours de la période de reproduction: premiers resultants. *In*: Boucaud-Camou, E, editor. “The cuttlefish” act. Proceedings of the first international Symposium on the cuttlefish *Sepia*. Caen: *Centre de Publications de l’Université de Caen*, p.167-77.

Lopes, F.J.and Dias, J.M. (2007). Residual circulation and sediment distribution in the Ria de Aveiro lagoon, Portugal. *Journal of Marine Systems*, 68: 507-528.

Mangold, K. and Bidder, A.M. (1989). Reproduction, croissance et durée de vie. InGrassé PP, editor. Traité de Zoologie, Tome V. *Céphalopodes*, Paris:Masson *In*: Bustamante, P., Teyssié, J., Fowler, S.W., Cotret, O., Danis, B., Miramand, P., Warnau, M. (2002b). Biokinetics of zinc and cadmium accumulation and depuration at different stages in the life cycle of the cuttlefish *Sepia officinalis. Marine Ecology Progress Series*, 231: 167-177.

Martin, J. and Flegal, A. (1975). High copper concentrations in squid levers in association with elevated levels of silver, Cd, and Zn. *Marine Biology*, 30: 51-55.

Miramand, P. and Guary, F.M. (1980). High concentrations of some heavy metals in tissues of the Mediterranean octopus. *Bulletin of Environmental Contamination and Toxicology*. 24: 783-788.

Miramand, P. and Bentley, D. (1992). Concentration and distribution of heavy metals in tissues of two cephalopods, *Eledone cirrhosa* and *Sepia officinalis*, from the French coast of the English Channel. *Marine Biololgy*, 114: 407-414.

Miramand, P., Bustamante, P., Bentley, D., Kouéta, D. (2006). Variation of heavy metal concentrations (Ag, Cd, Co, Cu, Fe, Pb, V, Zn) during the life cycle of the common cuttlefish *Sepia officinalis*. *Science of the Total Environment*, 361: 132-143.

Monteiro, L.R., Porteiro, F.M., Gonçalves, J.M. (1992). Inter- and intraspecific variation of mercury levels in muscle of cephalopods from the Azores. *Arquipélago Life Earth Sciences*, 10: 13-22.

Nixon, M. and Mangold, K. (1998). The early life of *Sepia officinalis*, and the contrast with that of *Octopus vulgaris* (Cephalopoda). *Journal of Zoology*, 245: 407-421.

Pastorinho, M.R., Telfer, T.C., Nogueira, A.J.A., Soares, A.M.V.M., Ranville, J.M. (2012). An evaluation of trace metal distribution, enrichment factors and risk in sediments of a coastal lagoon (Ria de Aveiro, Portugal). *Environmental Earth Sciences*, 67(7): 2043-2052.

Pereira, P. (2003). Repartição de metais e organoclorados em *Sepia officinalis* de dois sistemas lagunares: Ria de Aveiro e Ria Formosa. Tese de Mestrado, Universidade de Aveiro.

Picado, A., Dias, J.M., Fortunato, A. (2010). Tidal changes in estuarine systems induced by local geomorphologic modifications. Continental Shelf Research, 30(17): 1854-1864.

Pierce, G.J., Stowasser, G., Hastie, L.C., Bustamante, P. (2008). Geographical, seasonal and ontogenetic variation in cadmium and in mercury concentrations in squid (Cephalopoda: Teuthoidea) from UK waters. *Ecotoxicology and Environmental Safety*, 70: 422-432.

Pombo, L. (2005). Diversity, population dynamics and fish production capacity in a coastal lagoon – the Ria de Aveiro. Ph.D. Thesis, University of Aveiro, pp. 272.

Raimundo, J., Caetano, M., Vale, C. (2004). Geographical variation and partition of metals in tissues of *Octopus vulgaris* along the Portuguese coast. *Science of the Total Environment*, 325: 71-81.

Renzoni, A., Bacci, E., Falciai, L. (1973). Mercury concentration in the water, sediments and fauna of an area of the Tyrrhenian coast. *Revue Internationale d'Oceanographie Medicale,* 31-32: 17-45.

Richard, A. (1971). Contribution à l´´etude expérimentale de la croissance et de la maturation sexuelle de *Sepia officinalis* L. (Mollusque céphalopode). PhD thesis, University of Lille, France.

Robbe, D. (1984). Interprétation des teneurs en elements métalliques associés aux sediments. Rapport des laboratories, serie: *Environment et Génie Urbain* EG-1, 199:255-270 *In*: Fichet D., Boucher G., Radenac G., Miramand P. (1999). Concentration and mobilization of Cd, Cu, Pb and Zn by meiofauna populations living in harbor sediment: their role in the heavy metal flux from sediment to food web. *The Science of the Total Environment*, 243/244: 263-272.

Seixas, S., Bustamante, P., Pierce, G. (2005). Accumulation of mercury in the tissues of the common octopus *Octopus vulgaris* (L.) in two localities on the Portuguese coast. *Science of the Total Environment*, 340: 113-122.

Smith, J.D., Plues, L., Heyraud, M., Cherry ,R.D. (1984). Concentrations of the elements Ag, Al, Ca, Cd, Cu, Fe, Mg, Mn, Pb and Zn, and the radionuclides ^{210}Pb and ^{210}Po in the digestive gland of the squid *Notodarus gouldi*. *Marine Environmental Research*, 13: 55-68.

Vaz, N., Miguel, Dias, J., Chambel, Leitão, P. (2009). Three-dimensional modelling of a tidal channel: the espinheiro Channel (Portugal). *Continental Shelf Research*, 29: 29-41.

Valentim, J.M., Vaz, N., Silva, H., Duarte B., Caçador, I., Dias, J.M. (2013). Tagus estuary and Ria de Aveiro salt marsh dynamics and the impact of sea level rise. *Estuarine, Coastal and Shelf Science*, 1-14.

Villanueva, R. and Bustamante, P. (2006). Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. *Aquaculture*, 261: 225-240.

CHAPTER VI

Final remarks and Conclusions

VI. General Discussion and Conclusions

This study focus on the distribution patterns, the biology and some reproductive aspects of the life cycle of cuttlefish specie in Ria de Aveiro (chapter I). Due to the importance of cuttlefish, in diet and local economy of Aveiro region, the accumulation of some essential (Fe, Cu and Zn) and non-essential (Cd and Pb) metals in two target tissues (mantle and digestive gland) was studied (chapter II). Due to its extreme toxicity, strong capacity to persist in aquatic ecosystems and to the historical discharges of the chlor-alkali industry in Ria de Aveiro, mercury levels, in mantle and digestive gland, were studied separately from other mentioned metals in chapter III. Other demanding aspect was to investigate the patterns of accumulation of all above mentioned metals (Fe, Cu, Zn, Cd, Pb and Hg) as well as their seasonal variation with age in chapter IV.

Cuttlefish, *Sepia officinalis,* is an economically important target specie for Portuguese fisheries (Serrano, 1992), namely for Ria de Aveiro (Docapesca – Statistical Department, 2013). This work was performed at Ria de Aveiro (Aveiro lagoon) that is characterized by a large number of other smaller channels between which lie significant intertidal areas, essentially mudflats, salt marshes and old salt pans (Picado *et al*., 2010). Aveiro Lagoon is affected by a vast number of anthropogenic pressures: a) population settlement, the municipalities encircling this system are populated by 350,000 inhabitants (INE, 2011); b) industries (chemical, metallurgic, ceramics, tannery and pulp milling (Pacheco *et al*., 2005); c) agriculture and cattle rearing (Vasconcelos *et al*., 2007); c) offshore fisheries and fish and shellfish capture inside the lagoon (Pastorinho *et al*., 2012); d) the sizeable number of abandoned mines draining to the Vouga River or its tributaries (Delgado *et al*. 2000). Anthropogenic factors affect water quality and also influence the recruitment of several species in the lagoon (especially the migratory species like cuttlefish). Historical discharges of industrial and domestic effluents in Ria de Aveiro (Hall, 1982; Pereira *et al.*, 2009a), for many years, affected the community of aquatic individuals that live in the wide diversity of characteristic habitats of this aquatic systems (Pombo and Rebelo, 2002).

Several factors should be considered when studying the distribution of organisms such as seasonality, catchment size, estuary size and type, habitat type, near shore marine conditions, physical constrictions, the occurrence and severity of floods, salinity gradients, the ability of species to adjust to salinity and temperature fluctuations, dissolved oxygen levels, turbidity, food availability, predation, competition, reproductive conditions and habitat degradation (pollution and dredging) (Whitfield, 1996). Some of these factors cannot be achieved under field conditions, and in this study, abiotic factors like water temperature, dissolved oxygen, turbidity, pH and salinity were measured to comprehend cuttlefish distribution in Ria the Aveiro. Among the five factors studied, the two factors that seemed to be relevant to determine the presence or the absence of cuttlefish in the lagoon were the salinity and depth.

Salinity fluctuations in Ria the Aveiro depend mainly upon the balance between freshwater inflow and the tidal regime, with evaporation playing a major role in lagoon systems with a large surface area. The results obtained in this work suggest that, the cuttlefishes in Ria the Aveiro are therefore more tolerant to higher than lower salinity conditions, which is in close agreement with values obtained for optimum embryonic development and growth of young cuttlefish (36-37), under aquaculture conditions (Domingues *et al.*, 2001; Domingues *et al.*, 2004; Baeza-Rojano *et al.*, 2010). Indeed, the entrance of cuttlefish in the lagoon may delay or suffer an interruption due to the presence of freshwater (Jorge and Sobral, 2004). This is relevant because within the lagoon some areas are affected by freshwater flooding (especially the sites near the main rivers) and others by marine tides, whereas salinity is typically of marine environments.

Areão (ARE), the site without cuttlefishes, is the site located further away from the sea, so exhibits only minor tidal effects and also receives freshwater from lagoons (located to the South) through a system of ditches whose input is calculated as 1.9 m^3 s^{-1} (Rodrigues *et al*. 2009), both factors contribute to a very low salinity. This site presents the lower limits of salinity and depth, what may justify the absence of cutlefish.

Marine tide regulates the salinity in Aveiro lagoon but also influences the depth of sites, mainly the sites near the mouth of the lagoon. Indeed the sites that presented the highest values of depth were POR, SJA and GAF, all near the sea.

The abiotic characteristics observed seem to favor the colonization of the lagoon by cuttlefish, practically throughout the year, although with marked fluctuations in abundance.

S. officinalis reproduces only once, over a short period at the end of its life (Mangold, 1963). One of the most important characteristic of its life cycle is the seasonal migrations between shallow waters in summer and deeper waters waters in winter (Gauvrit *et al.*, 1997). Cuttlefish live approximately two years and exhibit mass mortality of adults after spawning period (Boletzky, 1983). Abiotic parameters such as salinity, temperature, dissolved oxygen, turbidity, etc., affect the development of the *Sepia officinalis* in its life cycle (Richard, 1971; Palmegiano and D'apote, 1983; Bouchaud and Daguzan, 1990; Domingues *et al.*, 2001). Temperature clearly plays a major role in determining the life span of *S. officinalis* (Richard, 1971; Forsythe *et al.* 1991, 1994).

The life cycle of cuttlefish in Ria de Aveiro starts with the entrance of adults entering reproduction period in spring. The reproduction peak is reached in summer, when water temperature begins to increase, and apparently ends with the death of post-spawning individuals and later in autumn with the return of new borned cuttlefish to sea. This structure of reproductive life cycle presented in the Ria de Aveiro is in accordance to others geographical areas, like the English Channel (Dunn, 1999; Wang *et al.*, 2003 and Challier *et al.*, 2005); the Estuary of Sado in Setúbal (Portugal) (Neves *et al.*, 2009) and the French coast (Morbihan Bay) (Blanc *et al.*, 1998).

The length of mature individuals caught in Ria de Aveiro suggests that cuttlefish breeders were in their first year of life. The energy required for the sexual development of males and females of cuttlefish is quite different (Dunn, 1999). Males attain sexual maturity earlier than females (Gauvrit *et al.*, 1997) being usually smaller than females (Pinczon-du-Sel and Daguzan, 1997). These facts explain the significant differences between weight-length relationships of different sexes in cuttlefish. Richard (1971) and Boletzky (1979) showed that reared cuttlefish growth was sigmoid. Forsythe and Van Heukelem (1987) described a two – phase curve, with an exponential phase followed by a logarithmic phase.

Cephalopods are known for their ability to accumulate high levels of essential elements and non-essential elements (Martin and Flegal, 1975; Miramand and Bentley, 1992; Bustamante *et al.*, 1998a, b; Bustamante *et al.*, 2000; Raimundo *et al.*, 2004). Considering these two facts, the historical problem of pollution of Ria de Aveiro and the strong capacity of cuttlefish to accumulate exogenous compounds, this study tried to understand the patterns of metal accumulation in *S. officinalis* in Ria de Aveiro.

One of the most important metals presented in the aquatic systems is mercury (Hg), since it is widely considered to be among the top priorities in environmental pollutants in the scope of the European Water Framework Directive (WFD) and at a global scale (Pereira *et al.*, 2009b). Ria de Aveiro is not an exception regarding the importance of this metal within the lagoon, in fact the studied area has historical discharges of effluents from the chlor-alkali industry of the industrial complex of Estarreja into

Laranjo bay (Lucas *et al.*, 1986; Hall *et al.*, 1985; Pereira *et al.*, 1998; Monterroso *et al.*, 2003b). Nowadays, the Laranjo bay is a non-significant active source of Hg since the discharges stopped in 1985, however it is still an internal source of Hg due to the sediments that have been highly contaminated with Hg (Hall *et al.*, 1985; Coelho *et al.*, 2008). Hg distribution in Ria de Aveiro showed that levels of Hg in the lagoon are low with the exception of Laranjo bay that presented higher values. However, only one individual of all the examined cuttlefishes presented an Hg level above the limit permitted, so we can conclude that the cuttlefish caught in the lagoon are not a concern for human health. Once more, abiotic parameters interfered with life of cuttlefish, because temperature can contribute to a higher methylation rate (Bustamante *et al.*, 2006a), which could lead to a higher uptake of mercury by aquatic organisms. The historical contamination of Laranjo bay and the higher values of temperature observed in this sampling site can explain the higher values of Hg in this specific area of the Ria de Aveiro. The Hg contained in the sediments may become available to biota (Coelho *et al.*, 2007), eventually to food chain thru the benthic organisms (Bryan and Langston, 1992; Lee *et al.*, 1998; Warren *et al.* 1998), or be remobilized when sediments are dredged and disposed into water bodies (Newell *et al.*, 1998; Chapman *et al.*, 2003).

Similarly the high Cd concentrations in Ria de Aveiro is also the result of the industrial complex of Estarreja, near the Laranjo bay, and the contamination of others locals of the lagoon are made by transport of contaminated sediments and water along the lagoon by the tide currents of Ria the Aveiro (Pereira *et al.*, 1998b, Monterroso *et al.*, 2003a) and by plankton exportation (Monterroso *et al.*, 2003b). Bustamante *et al.* (2000) suggested that high Cd levels in digestive gland of species captured in contaminated areas are also related to the environment.

Metal concentrations increase as the distance to the sea increase. This pattern of metals distribution in digestive gland appears to reflect the location of anthropogenic sources of metals discharged in the northern region of Ria de Aveiro (Monterroso *et al.*, 2003a). Laranjo (LAR) and Torreira (TOR) are, probably, the most contaminated areas, presenting the highest values of essential (Fe, Zn, Cu) and non-essential (Cd and Pb) metals in the digestive gland. The high values in Torreira (TOR) suggest that this site suffers the influence of Largo da Coroa, considered the second most contaminated area after Laranjo bay in Ria de Aveiro (Monterroso *et al.*, 2003a).

For metal-metal relationship in digestive gland and mantle, only Pb presented a low correlation between the two tissues. Only the ratio Cd:Cu in digestive gland presented a positive correlation with wet body weight, meaning that Cd was progressively sequestrated in digestive gland with age. This pattern was also observed for *S. officinalis* in Pereira *et al*. (2009b) and for *O. vulgaris* in Raimundo *et al*. (2005).

The non-essential metal Cd, when in high concentrations in digestive gland, can compete with Zn and Cu (essential metals) for the same ligands, specific metalloproteins presented in digestive gland of cephalopods, since they have similarities in chemical behaviour (Miramand and Bentley, 1992; Bustamante, 1998a). For this reason, significant correlations between Cd-Cu and Cd-Zn were expected, as found in Pereira *et al*. (2009b), although only Cd and Cu in digestive gland showed the expected behaviour. Since essential elements like Cu tend to be maintained at a fairly constant concentrations in tissues, Cd:Cu ratios increase in cephalopods contaminated by Cd (Bustamante *et al*., 1998a; Raimundo *et al*., 2005; Pereira *et al*., 2009b). Measured levels of Cd in cuttlefish tissues, despite being below legal limits, may lead to some concern for human consumption.

The cuttlefish *S. officinalis* feeds on a large variety of living prey, including small molluscs, crabs, shrimps, other cuttlefish, and juvenile demersal fishes. Crustaceans and fishes, which are preferential preys of cuttlefish (Pinczon du Sel *et al*., 1997; Pinczon du Sel *et al*., 2000), also accumulate more Hg than other species (Schuhmacher *et al*., 1994).

Mercury is considered to be one of the most problematic metals in marine ecosystems due to bioaccumulation and biomagnification in marine food webs (EPA, 2001).

Various studies reported Hg concentrations in different tissues (Alcobia, 1995; Buzina *et al*., 1989; Stoeppler *et al*., 1979; Rossi *et al*., 1993; Seixas *et al*., 2005a; Raimundo *et al*., 2004, 2010; Bustamante *et al*., 2008). Mercury concentrations in the Aveiro lagoon are greatly influenced by the historical discharges of mercury from industrial activities into Laranjo Bay (Pereira *et al*., 1998a; Pereira *et al*., 2009a), leading to Hg concentrations higher than other ecosystems also polluted, like Kastela Bay in Adriatic that is known for receiving discharges of Hg from mine exploitation (Faganeli *et al*., 2003).

Like other metals, Hg concentrations in cephalopod may vary with biological factors such as age (size), sex and maturity stage (Bustamante *et al*., 1998a; Raimundo *et al*., 2004; Seixas *et al*., 2005a; Ayas and Ozogul, 2011). It is generally admitted that Hg concentrations in cephalopod tissues are positively correlated with size (Monteiro *et al*., 1992; Rossi *et al*., 1993; Storelli and Marcotrigiano, 1999 in Bustamante *et al*., 2006a), however, due to restrict range of size of captured individuals this couldn't be verified.

While the mantle is a body compartment that can store Hg in significant amounts, the digestive gland plays a major role in storage and detoxification of several metals. The different patterns of accumulation of trace metals and Hg suggest that cephalopods have different mechanisms/rates of uptake and/or sequestration of these metals (Pierce *et al*., 2008). This contrast between trace metals and Hg partitioning was also observed in other cephalopods (Raimundo *et al*., 2004; Seixas *et al*., 2005a; Bustamante *et al*., 2006a).

Metal bioaccumulation in cephalopods is usually related to a single organ, mainly the digestive gland, known to play a major role in the energetic metabolism of cephalopods (Boucaud-Camou and Boucher-Rodoni, 1983 in Bustamante *et al*., 2006a). The digestive gland is known to accumulate metal in large amounts (Martin and Flegal, 1975; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante *et al*., 2000). This organ is deeply involved in the metabolism of Zn, Cu and Cd (Martin and Flegal, 1975; Smith *et al*., 1984; Finger and Smith, 1987; Miramand and Guary, 1980; Bustamante *et al*., 1998b, 2002a). The mean concentrations of Fe, Zn, Cu, Cd and Pb in the digestive gland are much higher than the mean concentrations of those metals in mantle while Hg presents similar levels in both tissues. Metal concentrations in different maturity stages of cuttlefish show that the accumulation is different for each stage of their life cycle (Bustamante *et al.*, 2002b; Bustamante *et al.*, 2004; Bustamante *et al.*, 2006b; Villanueva and Bustamante, 2006). The results obtained in this studied determine that some metals remain constant along the age (Fe and Cu); others increase along maturity stage (Cd, Zn and Hg) and some decrease with age (Pb). Seasonal migrations evidenced in the life cycle of *S.* officinalis may interfere with bioaccumulation processes of metals, since cuttlefish migrates from coastal areas (more polluted) to offshore (less polluted than coast) (Miramand *et al.*, 2006).

In digestive gland, Zn presented a significant increase from stage I (243±112 µg g-1.DWt) to stage II (578±270 µg g-1.DWt), stage I presented a value 2.4 times higher than stage II. This increase between immature and juveniles could be due to differences in efficiency of their digestive metabolism (Mangold, 1989 in Bustamante *et al*., 2002b).

The levels of Fe, Zn, Cu, Cd and Pb in the digestive gland are higher than those found in mantle, what is in close agreement with other cephalopod species (Raimundo *et al*., 2004; Raimundo *et al*., 2005; Pereira, 2003; Pereira *et al*., 2009b). These metals, in *S. officinalis* are preferentially accumulated in the digestive gland. Various studies confirmed that the digestive gland of cephalopods concentrates high levels of Zn and Cu, and have an exceptional ability to store Cd (Miramand and Guary, 1980; Bustamante *et al*., 1998b, 2002a).

In general, metal concentrations measured in this study for mantle and digestive gland of *S. officinalis* were higher, when compared with values reported for same species and other cephalopods of various studies, but comparable to previous studies along the Portuguese coast (Pereira *et al*., 2009b; Seixas *et al*., 2005b; Raimundo *et al*., 2005, 2008). Nevertheless, comparisons between specimens from different ecosystems must be done carefully as identical species may exhibit different feeding habits, growth rates and habitats, which may interfere with metal accumulation (Bustamante *et al*., 1998b; Raimundo *et al*., 2004; Pierce *et al*., 2008).

Extremely high values of Cd were found in the mantle of specimens captured in Laranjo (LAR) but a relatively low value of this element was found in the digestive gland. The increase of Cd in mantle, without a corresponding increase in the digestive gland, where this element is stored (Bustamante, 1998; Bustamante *et al*., 2002a; Bustamante *et al*., 2002b) suggests that the levels in mantle result from a higher transference of Cd in water, most likely as a result of uptake through the diet (Bustamante, 1998; Bustamante *et al*., 2002b). For Cd the values obtained in all sites of Ria de Aveiro are higher than the values measured in previous studies.

The similarity of Hg levels in mantle and digestive gland could be explained by the affinity of mantle to store MeHg, i.e. predominant form of Hg stored in muscle tissue (Harris *et al*., 2003; Amlund *et al*., 2007). However, compared to other metals like Cd, the role of digestive gland in storage of Hg appears to be relatively limited. This may be due to a preferential redistribution of Hg to muscular tissues as methylmercury (MeHg) (Bustamante *et al*., 2006a).

The most striking result was the extremely high value of Pb in the digestive gland of immature specimens. This aspect can be related with the source of intake of this metal. To the best of our knowledge, no data on the respective proportions of elements incorporated from food and seawater has been published for cephalopods. So we consider that there were two possibilities for this value, the food ingested by immature specimens is probably highly contaminated with Pb or the specimens were subjected to an acute exposure and recent uptake of Pb by seawater. On these immature specimens, being on an early stage of maturation, the digestive gland may not be working properly, leading only to the accumulation of metal and not to its detoxification. It was shown (Miramand *et al*. 2006) that embryos present very low metal concentrations compared to older cuttlefish, and that concentrations of embryos were close or even identical to those measured in the vitellus of newly spawned eggs, which suggests that the vitellus contains a sufficient amount of essential metals (Cu, Fe, and Zn) needed for the development of the embryos.

During the life cycle of cuttlefish, specifically between stage I and stage IV, Cd concentrations increased 14 times, in the digestive gland, compared to a factor of 2 for mantle. Cd is strongly retained in the tissue of adult cuttlefishes and this high retention capacity concerned almost exclusively the digestive gland. Several authors reported that the digestive gland play a primary role in the accumulation of Cd in cuttlefish (Miramand and Bentley, 1992; Bustamante, 1998; Bustamante *et al*., 2002a). Thus, assimilated Cd in the digestive gland may be considered as actually stored. The lower retention efficiency for Cd measured in immature cuttlefish would result from the incomplete development of the digestive gland in early juveniles (Bustamante *et al*., 2002b) and a restrict transfer of this non-essential metal from food in earlier stages. Cephalopods such as *Sepia officinalis* accumulate Cd mainly from diet (Bustamante *et al*., 2002b).

The results revealed that the digestive gland of *S. officinalis* has an extraordinary capacity to store harmful elements like Cd and Pb, but also essential elements like Fe, Zn and Cu. Several studies also confirmed that the digestive gland of cephalopods concentrates high levels of Zn and Cu, and have an exceptional ability to store Cd (Miramand and Guary, 1980; Bustamante *et al*., 1998b, 2002b). However, the digestive gland presented other functions like redistribution of metals to others tissues, and for these reasons the digestive gland is consider a good biomarker of contamination for metals in cuttlefish. Due to the species mobility together with the influence of local food composition at different sites of the lagoon, metal concentrations in tissues cannot be exclusively viewed as direct reflex of environmental conditions. However, the obtained results and all that has been published suggest that the major source of contamination in Ria de Aveiro is the large complex of chemical industries located near the lagoon. The continuous effluent discharge had a considerable impact on bottom sediments (OSPAR, 2000). Sediments act as source and sink for pollution, because once the pollutants are introduced, they may remain accumulated in soil for several years, even after removal of the sources (Paterson *et al*., 1996; Hursthouse, 2001; Wong *et al*., 2006). For example, Ria de Aveiro still have a gradient contamination in the sediments (Pereira *et al*., 1998 and Coelho *et al*., 2005) because of the continuous direct discharges, since the 1950s of the chlor-alkali industry. Soil contamination resulting from anthropogenic activities may derive from both diffuse and local sources and is generally reflected by enhanced concentrations of acidifying contaminants (e.g. SO2, NOx), metals (e.g. cadmium, lead, mercury), metalloids such as arsenic, and organic compounds (such as pesticides, herbicides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins (PCDDs) (EC, 2002). According to Scullion (2006) the main anthropogenic sources of soil contaminants are mining and smelting; fossil fuel combustion; sewage sludge; process and manufacturing industries (specifically metallurgical, electronics and chemical); waste disposal; the land spreading of fertilizers, fungicides and other agricultural materials; atmospheric deposition from traffic; waste incineration; the spillage of liquids such as solvents or oil; and practices of irrigation with contaminated waters. For Aveiro Lagoon previous studies (Pastorinho *et al*., 2012) considered that the major sources of sediments contamination are the 3 main watercourses (Caster, Antuã, and Vouga). However, Ria de Aveiro is influenced by strong marine tides (70 x 106 m^3, in Picado *et al*. 2010), which redistributes sediment bound metals. According to Pastorinho *et al.* (2012), bottom sediments in the Ria de Aveiro system are in their majority unpolluted, with few metal hotspots and only these sites constitute a risk for biota. These results are in accordance with the ones obtained in this work, which demonstrated that the only problematic site were Laranjo bay, presenting much higher values of metals. Indeed, these results suggest a gradient of contamination in the direction of Laranjo.

As final conclusion, as shown throughout the present study, there are general similarities but also differences between the Ria de Aveiro and other geographical areas referred in the study. This emphasizes its uniqueness not only in terms of abiotic parameters but especially in the biotic aspects, which enhances the idea that this transitional ecosystem should be deeply studied for this specie. Indeed the Ria the Aveiro is the only typically estuarine coastal lagoon, all the others sites represented the offshore or presented a higher communication with the offshore.

This study has provided the first attempt to explain the seasonal and spatial variations in the reproductive life cycle of the cuttlefish in a wide range of sites in the Ria de Aveiro, having in consideration historical discharges in the lagoon, and the results revealed different patterns in the accumulation of different metals along the life cycle.

Future research should be addressed in order to enable a progressive understanding of the role of cuttlefish in Ria de Aveiro. New studies focused on the trophic ecology for cuttlefish should be made to better understand the predatory impact of this specie in fish community of the lagoon.

VI.1. References

Alcobia, H. (1995). Biologia e acumulação de mercúrio no choco *Sepia officinalis* (Linnaeus, 1758) do Estuário do Sado. Relatório de estágio de licenciatura.

Amlund, H., Lundebye, A.-K., Berntssen, M.H.C. (2007). Accumulation and elimination of methylmercury in Atlantic cod (*Gadus morthua* L.) following dietary exposure. *Aquatic toxicology*, 83: 323-330.

Ayas, D. and Ozogul, Y. (2011). The effects of Season and sex in the Metal Levels of Mature Common Cuttlefish (*Sepia officinalis*) in Mersin Bay, Northeastern Mediterranean. *Journal of food Science,* Vol.76:4.

Baeza-Rojano, E., García, S., Garrido, D., Guerra-García, J.M., Domingues, P. (2010) Use of Amphipods as alternative prey to culture cuttlefish (*Sepia officinalis*) hatchlings. *Aquaculture*, 300: 243–246

Blanc, A., Pinczon du Sel, G. and Daguzan, J. (1998). Habitat and diet of early stages of *Sepia officinalis* L. (Cephalopoda) in Morbihan Bay, France. *Journal of Molluscan Studies*, 64: 263-274.

Boletzky, S.V. (1979). Growth and life-span of *Sepia officinalis* under artificial conditions. *Rapport Comunitaire International Mer Mèditeranee*, 10, 10 p.

Boletzky, S.V. (1983). *Sepia officinalis*. *In*: Boyle, P.R. (Ed.), Cephalopod Life Cycles, vol.1. *Academic Press*, London, pp.31-52.

Boucaud-Camou, E. and Boucher-Rodoni, R. (1983). Feeding and digestion in Cephalopods. In: Saleudin A.S.M., Wilbur K.M., editors. The Mollusca, vol. 5, Part.2 New York: *Academic Press*: p.149-87. *In*: Bustamante, P., Lahaye, V., Durnez, C., Churlaud, C., Caurant, F. (2006a). Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environment*, 368: 585-596.

Bouchand, O. and Daguzan, J. (1990). Etude expérimentale de l'influence de la temperature sur le déroulement du développement embryonnaire de la Seiche *Sepia officinalis* L. (Céphalopode, *Sepioidae*). *Cahiers de Biologie Marine*, 31: 131-145.

Bryan, G.W. and Langston, W.J. (1992). Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution*, 76: 89-131.

Bustamante, P., Caurant, F., Fowler, *S.*, Miramand, P. (1998b) Cephalopods as a vector for the transfer of Cd, to top marine predators in the North-East Atlantic Ocean. *Science of the Total Environment*, 220: 71-80.

Bustamante, P., Cherel, Y., Caurant, F., Miramand, P. (1998a) Cadmium, copper and Zinc in octopuses from Kerguelen Islands, Southern Indian Ocean. *Polar Biology*, 19: 264-271.

Bustamante, P., Grigioni, *S.*, Boucher-Rodoni, R., Caurant, F., Miramand, P. (2000). Bioaccumulation of 12 trace elements in the tissues of Nautilus *Nautilus macromphalus* from New Caledonia. *Marine Pollution Bulletin*, 40: 688-696.

Bustamante, P., Cosson, R.P., Gallien, I., Caurant, F., Miramand, P. (2002a). Cadmium detoxification processes in the digestive gland of cephalopods in relation to accumulated Cd concentrations. *Marine Environmental Research*, 53: 227-241.

Bustamante, P., Teyssié, J-L., Fowler, S.W., Cotret, O., Danis, B., Miramand, P., *et al.* (2002b). Biokinetics of zinc and cadmium accumulation and depuration at different stages in the life cycle of the cuttlefish *Sepia officinalis*. *Marine Ecology Progress Series*, 231: 167-77.

Bustamante, P., Teyssié, J.-L., Danis, B., Fowler, S.W., Miramand, P., Cotret, O., Warnau, M. (2004). Uptake, transfer and distribution of silver rand cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. *Marine Ecology Progress Series*, 269: 185-195.

Bustamante, P., Lahaye, V., Durnez, C., Churlaud, C., Caurant, F. (2006a). Total and organic Hg concentrations in cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environment*, 368:585-596.

Bustamante, P., Teyssié, J.L., Fowler, S.W., Warnau, M.. (2006b). Assessment of the exposure pathway in the uptake and distribution of americium and cesium in cuttlefish (*Sepia officinalis*) at different stages of its life cycle. *Journal of Experimental Marine Biology and Ecology*. 331, 198-207.

Bustamante, P, González, A.F., Rocha, F., Miramand, P., Guerra, A. (2008). Metal and metalloid concentrations in the giant squid *Architeuthis dux* from Iberian Waters. *Marine Environmental Research*, 66: 278-287.

Buzina, R., Suboticanec, K., Vukusic, J., Sapunar, J., Antonic, K., Zorica, M. (1989). Effect of industrial pollution onseafood contente and dietary intake of total and methylmercury. Science of the Total Environment, 78: 45-57.

Challier, L., Royer, J., Robin, J-P. (2002). Variability in age-at-recruitment and early growth in English Channel *Sepia officinalis* described with statolith analysis. *Aquatic Living Resources*, 15: 303-311.

Challier, L., Dunn, M.R., Robin, J.P. (2005). Trends in age-at-recruitment and juvenile growth of cuttlefish, *Sepia officinalis*, from the English channel. *ICES Journal of Marine Science*, 62: 1671-1682.

Chapman, P.M., Wang, F., Janssen, C.R., Goulet, R.R., Kamunde, C.N. (2003). Conducting ecological risk assessment of inorganic metals and metalloids: current status. *Human and Ecological Risk Assessment*, 9: 641-697.

Coelho, J.P., Pereira, M.E., Duarte, A., Pardal, M.A. (2005). Macroalgae response to a mercury contamination gradient in a temperate costal lagoon (Ria de Aveiro), Portugal). *Estuarine, Coastal and Shelf Science*, 65: 492-500.

Coelho, J.P., Policarpo, E., Pardal, M.A., Millward, G.E., Pereira, M.E., Duarte, A.C. (2007). Mercury contamination in invertebrate biota in a temperature coastal lagoon (Ria de Aveiro, Portugal). *Marine Pollution Bulletin*, 54: 464-488.

Coelho, J.P., Nunes, M., Dolbeth, M., Pereira, M.E., Duarte, A.C., Pardal, M.A. (2008) The role of two sediment dwelling invertebrates on the mercury transfer from sediments to the estuarine trophic web. *Estuarine, Coastal and Shelf Science*, 78(3): 516-523.

Delgado, H., Rocha, F., Gomes, C. (2000). Modelos de dispersão de metais pesados nos sedimentos recentes da laguna de Aveiro e sua relação com a composição e textura dos sedimentos. *In*: Duarte, A., Vale, C., Prego, R. (eds) Estudos de Biogeoquímica na Zona Costeira Ib+erica (actas IX Seminário Ibérico de química Marinha), Universidade de aveiro, Portugal, pp 325-336.

Dias, J.M., Picado, A. (2011). Impact of morphologic anthropogenic and natural changes in estuarine tidal dynamics. *Journal of Coastal Research*, 64: 1490-1494.

DocaPesca of Aveiro. (2013). Total landings of cuttlefish in Aveiro Lagoon by tones captured and annual turnover per year, since 2002 to 2012. Statistical Department.

Domingues, P.M., Kingston, T., Sykes, A., Andrade, J.P. (2001). Growth of young cuttlefish, *Sepia officinalis* (Linnaeus, 1758) at the upper end of the biological distribution temperature range. *Aquaculture Research*, 32: 923-930.

Domingues, P., Sykes, A., Sommerfield, A., Almansa, E., Lorenzo, A., Andrade, J.P. (2004). Growth and survival of cuttlefish (Sepia officinalis) of different ages fed crustaceans and fish. Effects of frozen and live prey. *Aquaculture*, 229: 239-254.

Dunn, M.R. (1999) Aspects of the stock dynamics and exploitation of cuttlefish, *Sepia officinalis* (Linnaeus, 1758), in the English Channel. *Fisheries Research*, 40: 277-293.

EC European Commission. (2002). Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbonsin food. Document SCF/CS/CNTM/PAH/29 Final, available on http://europa.eu.int/comm/food/fs/sc/scf/index_en.html.

Elliot, M. and Hemingway, K.L. (Eds.) (2002). *Fishes in Estuaries*. Blackwell Science, Oxford.

EPA - United States Environmental Protection Agency. (2001). Water quality criterion for the protection of human health: methylmercury. *Office Science and Technology, Office of Water.* Washington, 253p.

Faganeli, J., Horvat, M., Covelli, S., Fajon, V., Logan, M., Lipej, L., Cermelj, J. (2003). Mercury and methylmercury *in* the Gulf of Trieste (Northern Adriatic Sea). *Science of the Total Environmenl,* 304: 315-326.

Finger, J.M and Smith, J.D. (1987). Molecular association of Cu, Zn, Cd and ^{210}Po in digestive gland of the squid *Nototodarus gouldi. Marine Biology,* 95: 87-91.

Forsythe, J.W. and Van Heukelem, W.F. (1987). Growth. *In:* Cephalopod life Cycles, vol II: Comparative Reviews, pp. 135-156. Ed. by P.R. Boyle *Academic Press*, London, 441pp.

Forsythe, J.W., Hanlon, R.T., DeRusha, R.H. (1991). Pilot large-scale culture of *Sepia officinalis* in biomedical research. *In*: Boucaud-Camou E. (ed.), *The cuttlefish*. Centre de publications de l'Université de Caen, pp. 313-323.

Forsythe, J.W., DeRusha, R.H., Hanlon, R.T. (1994). Growth, reproduction and life span of *Sepia officinalis* (Cephalopoda: Mollusca) cultured through seven consecutive generations. *Journal of Zoology*, London, 233: 175-192.

Gauvrit, E., Le Goff, R., Daguzan, J. (1997). Reproductive cycle of the cuttlefish, *Sepia officinalis* (L.) in the Northern part of the Bay of Biscay. *Journal of Molluscan Studies*, 63: 19-28.

Hall, A. (1982). Water quality problems in Ria de Aveiro. In: Actual Problems of Oceanography in Portugal, Junta Nacional de Investigação científica e Tecnológica and Nato Marine Sciences Panel (ED.), Lisboa, pp.159-169.

Hall, A., Lucas, M.F., Caldeira, M.T., Duarte, A.C. (1985). Distribuição de Mercúrio nos Sedimentos da Ria de Aveiro (Distribution of Mercury in the sediments of Ria de Aveiro, Portugal). Actas das jornadas da Ria de Aveiro. Poluição da Ria de Aveiro, I. Câmara de Aveiro, pp.103-25.

Harris, H.H., Pickering, I.J., George, G.N. (2003). The chemical form of mercury in fish. *Science*, 301: 1203.

Hursthouse, A.S. (2001). The relevance of speciation in the remediation of soils and sediments contaminated by metallic elements – an overview and exemples from Central Scotland, UK. *Journal of Environmental Monitoring*, 3: 49-60.

INE (2011) Instituto Nacional de Estatistica. Censos 2011: resultados provisórios.

Jorge, I. and Sobral, M. P. (2004). Alguns aspectos da biologia e da ecologia do choco, *Sepia officinalis* (Linnaeus, 1758), da região de Aveiro. *Relatório Técnico-Científico do IPIMAR*, *Série digital* (http://ipimar-iniap.ipimar.pt), nº15, 29 p.

Lee, C., L., Fang, M.D., Hsieh, M.T. (1998). Characterization and distribution of metals in surficial sediments in Southwestern Taiwan. *Marine Pollution Bulletin*, 36(6): 464–471.

Lucas, M.F., Caldeira, M.T., Hall, A., Duarte, A.C., Lima, C. (1986). Distribution of mercury in the sediments and fishes of the lagoon of Aveiro, Portugal. *Water Science and Technology*., 18: 141-148.

Mangold-Wirz, K. (1963). Biologie des Céphalopodes bentiques et nectoniques de la Mer Catalane. *Vie Milieu*, suppl. 13: 1-285.

Mangold, K. and Bidder, A.M. (1989). Reproduction, croissance et durée de vie. InGrassé PP, editor. Traité de Zoologie, Tome V. *Céphalopodes*, Paris:Masson. *In*: Bustamante P., Teyssié J., Fowler S.W., Cotret O., Danis B., Miramand P., Warnau M. (2002b). Biokinetics of zinc and cadmium accumulation and depuration at different stages in the life cycle of the cuttlefish *Sepia officinalis*. *Marine Ecology Progress Series*, 231: 167-177.

Martin, J. and Flegal, A. (1975). High copper concentrations in squid levers in association with elevated levels of silver, Cd, and Zn. *Marine Biology*, 30: 51-55.

Miramand, P. and Guary, J.C. (1980). High concentrations of some heavy metals in tissuesof the Mediterranean octopus. *Bulletin of Environmental Contamination and Toxicology*, 24: 783-788.

Miramand, P. and Bentley, D. (1992). Concentration and distribution of heavy metals in tissues of two cephalopods, *Eledone cirrhosa* and *Sepia officinalis*, from the French coast of the English Channel. *Marine Biology*, 114: 407-414.

Miramand, P., Bustamante, P., Bentley, D., Kouéta, D. (2006). Variation of heavy metal concentrations (Ag, Cd, Co, Cu, Fe, Pb, V, Zn) during the life cycle of the common cuttlefish *Sepia officinalis*. *Science of the Total Environment*, 361: 132-143.

Monteiro, L.R., Porteiro, F.M., Gonçalves, J.M. (1992). Inter- and intraspecific variation of mercury levels in muscle of cephalopods from the Azores. *Arquipélago Life Earth Sciences*, 10: 13-22.

Monterroso, P., Pato, P., Pereira, E., Vale, C., Duarte, A.C. (2003a). Distribution and accumulation of metals (Cu, Cd, Zn and Pb) in sediments of a lagoon on the northwestern coast of Portugal. *Marine Pollution Bulletin*, 46: 1200-1211.

Monterroso, P., Abreu, *S*.N., Pereira E., Vale C., Duarte A.C. (2003b). Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro). *Acta Oecologica*, 24: S351-S357.

Neves, A., Cabral, H., Sequeira, V., Figueiredo, I., Moura, T., Gordo, L.S. (2009) Distribution patterns and reproduction of the cuttlefish *Sepia officinalis* in the Sado estuary (Portugal). *Journal of the Marine Biological Association of the United Kingdom*, 89 (3): 579-584.

Newell, R.C., Seiderer, L.J., Hitchcock, D.R. (1998). The impact of dredging works in coastal waters: a review of sensitivity to disturbance and subsequent recovery of biological resourses on the sea bed. *Oceanography and Marine Biology*, 36: 127-178.

OSPAR Commission (2000). Quality status Report 2000: Region IV – Bay of Biscay and Iberian Coast, OSPAR Commission, London. 134 + XIIIpp.

Pacheco, M., Santos, M.A., Teles, M., Oliveira, M., Rebelo, J.E., Pombo, L. (2005). Biotransformation and genotoxic biomarkers in mullet species (*Liza* sp.) from a contaminated coastal lagoon (Ria de Aveiro, Portugal). *Environmental Monitoring and Assessment*, 107: 133–153.

Palmegiano, G.B. and D'Apote, M.P. (1983). Combinated effects of temperatura and salinity on cuttlefish (*Sepia officinalis* L.) hatching. *Aquaculture*, 35: 259-264.

Pascual, E. (1978). Crescimiento y alimentacion de tres generaciones de *Sepia officinalis* en cultivo. *Investigacion Pesquera*, 42: 421-442.

Pastorinho, M.R., Telfer, T.C., Nogueira, A.J.A., Soares, A.M.V.M., Ranville, J.F. (2012). An evaluation of trace metal distribution, enrichment factos and risk in sediments of a coastal lagoon (Ria de Aveiro, Portugal). *Environmental Earth Science*, DOI: 10.1007/s12665-012-1643-xOnline First™.

Paterson, E., Sanka, M., Clark, L. (1996). Urban soils as pollutant sinks - a case study from Aberdeen, Scotland. *Applied Geochemistry*, 11: 129-131.

Pereira, M.E., Duarte, A.C., Millward, G., Abreu, S.N., Vale, C. (1998a). An estimation of industrial mercury stored in sediments of a confined area of the lagoon of Aveiro (Portugal). *Water Science and Technology*, 37(6-7): 125-130.

Pereira, M.E., Duarte, A.C., Millward, G., Vale, C., Abreu, *S*.N. (1998b). Tidal export of particulate mercury from the most contaminated area of Aveiro´s Lagoon, Portugal. *Science of the Total Environment*, 213: 157-163.

Pereira, M.E., Lillebø, A.I., Pato, P., Válega, J.P., Coelho, C.B., Lopes, C.B., Rodrigues, S., Cachada, A., Otero, M., Pardal, M.A., Duarte, A.C. (2009a). Mercury pollution in Ria de Aveiro (Portugal): a review of the system assessment. *Environmental Monitoring and Assessment*, 155(1-4): 39-49.

Pereira, P., Raimundo, J., Vale, C., Kadar, E. (2009b). Metal concentrations in digestive gland and mantle of *Sepia officinalis* from two coastal lagoons of Portugal. *Science of the Total Environment*, 407: 1080-1088.

Picado, A., Dias, J.M., Fortunato, A.B. (2010). Tidal changes in estuarine systems induced by local geomorphologic modifications. *Continental Shelf Research*, 30 (17), 1854–1864.

Pierce, G.J., Stowasser, A., Hastie, L.C., Bustamante, P. (2008). Geographical, seasonal and ontogenetic variation in cadmium and mercury concentrations in squid (Cephalopoda: Teuthoidea) from UK waters. *Ecotoxicology and Environmental Safety*, 70: 422-432.

Pinczon du Sel, G. and Daguzan, J. (1997). A note on sex ratio, length and diet of a population of cuttlefish *Sepia officinalis* L. (Mollusca:Cephalopoda) sampled by three fishing methods. *Fisheries Research*, 32: 191-195.

Pinczon du Sel, G., Blanc, A., Daguzan, J. (2000). The diet of the cuttlefish *Sepia Officinalis* L. (Mollusca:Cephalopoda) during its life cycle in the Northern Bay of Biscay (France). *Aquatic Sciences*, 61: 167-178.

Pombo, L. and Rebelo, J.E. (2002). Spatial and temporal organisation of a coastal lagoon fish community, Ria de Aveiro, Portugal. *Cybium*, 26(3): 185-196.

Pombo, L. (2005). Diversity, population dynamics and fish production capacity in a coastal lagoon – the Ria de Aveiro. Ph.D. Thesis, University of Aveiro. pp. 272.

Raimundo, J., Caetano, M., Vale, C. (2004). Geographical variation and partition of metals in tissues of *Octopus vulgaris* along the Portuguese coast. *Science of the Total Environment*, 325: 71-81.

Raimundo, J., Pereira, P., Vale, C., Caetano, M. (2005). Fe, Zn, Cu and Cd concentrations in the digestive gland and muscle tissues of *Octopus vulgaris* and *Sepia officinalis* from two areas in Portugal. *Ciencias Marinas*, 31(1B): 243-251.

Raimundo, J., Vale, C., Canário, J., Branco, V., Moura, I. (2010). Relations between mercury, methyl-mercury and selenium in tissues of *Octopus vulgaris* from the Portuguese Coast. *Environmental Pollution*, 158: 2094-2100.

Renzoni, A., Bacci, E., Falciai, L. (1973). Mercury concentration in the water, sediments and fauna of an area of the Tyrrhenian coast. *Revue Internationale d'Oceanographie Médicale*, 31-32: 17-45.

Richard, A. (1971). Contribution à l'étude expérimentale de la croissance et de la maturation sexuelle de *Sepia officinalis* L. (Mollusque: Céphalopode). Thèse de Doctorat d'État, Université de Lille, 243: 1-2264.

Rodrigues, M., Oliveira, A., Queiroga, H., Fortunato, A.B., Zhang, Y.J. (2009). Three-dimensional modeling of the lower trophic levels in the Ria de Aveiro (Portugal). *Ecological Modelling*, 220(9-10): 1274-1290.

Rossi, A., Pellegrini, D., Belcari, P., Barghigiani, C. (1993). Mercury in *Eledone cirrhosa* from the Northern Tyrrhenian Sea: contents and relations with life cycle. *Marine Pollution Bulletin*, 26(12): 683-6.

Schuhmacher, M., Batiste, J., Bosque, M.A., Domingo, J.L., Corbella, J. (1994). Mercury concentrations in Marine species from the coastal area of Tarragona Province, spain. Dietary intake of mercury through fish and seafood consumption. *Science of the Total Environment*, 156 (3): 269-273.

Scullion, J. (2006). Remediating polluted soils. *Naturwissenschaften*, 93:51–65.

Seixas, S., Bustamante, P., Pierce, G. (2005a). Accumulation of mercury in the tissues of the common octopus *Octopus vulgaris* (L.) in two localities on the Portuguese coast. *Science of the Total Environment*, 340: 113-122.

Seixas, *S*., Bustamante, P., Pierce, G.J. (2005b). Interannual patterns of variation in concentrations of trace elements in arms of *Octopus vulgaris*. *Chemosphere,* 59: 1113-1124.

Serrano, M. (1992). Contribuição para o conhecimento da biologia e pescaria do choco, *Sepia officinalis* (Linnaeus, 1758) no estuário do Sado e zona costeira adjacente. Relatório Técnico-Científico, INIP, Lisboa.

Smith, J.D., Plues, L., Heyraud, M., Cherry, R.D. (1984). Concentrations of the elements Ag, Al, Ca, Cd, Cu, Fe, Mg, Mn, Pb and Zn, and the radionuclides ^{210}Pb and ^{210}Po in the digestive gland of the squid *Notodarus gouldi*. *Marine Environmental Research*, 13: 55-68.

Stoeppler, M., NÜrnberg, H.W. (1979). Comparative studies on trace metal levels in marine biota: III. Typical levels and accumulation of toxic trace metals in muscle tissue and organs of marine organisms from different European seas. *Ecotoxicology and environmental Safety,* 3(4): 335-351.

Storelli, M.M., Marcotrigiano, G.O. (1999). Cadmium and total mercury in some cephalopods from the South Adriatic Sea (Italy). *Food Additives and Contaminants*, 16(6): 261-265. *In*: Bustamante P., Lahaye V., Durnez C., Churlaud C., Caurant F. (2006a). Total and organic Hg concentrations *in* cephalopods from the North Eastern Atlantic waters: Influence of geographical origin and feeding ecology. *Science of the Total Environment*, 368: 585-596.

Vasconcelos, R.P., Reis-Santos, -P., Fonseca, V, Maia, A., Ruano, M., França, S, Vinagre, C., Costa, M.J., Cabral, H.N. (2007). Assessing anthropogenic pressures on estuarine fish nurseries along the Portuguese coast: a multi-metric index and conceptual approach. *Science of the Total Environment*, 374: 199–215.

Villanueva, R. and Bustamante, P. (2006). Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. *Aquaculture*, 261: 225-240.

Wang, J., Pierce, G.J., Boyle, P.R., Denis, V., Robin, J.-P, Bellido, J.M. (2003). Spatial and temporal patterns of cuttlefish (*Sepia officinalis*) abundance and environmental influences – a case study using trawl fishery data in French atlantic coastal, English Channel, and adjacent waters. *ICES Journal of Marine Science*, 60: 1149-1158.

Warren, L.A., Tessier, A., Hare, L. (1998). Modelling Cd accumulation by benthic invertebrates in situ: the relative contributions of sediment and overlying water reservoirs to organism Cd concentrations. *Limnology and Oceanography*, 43 (7): 1442-1454.

Whitfield, A.K. (1996). A review of factors influencing fish utilization of South African Estuaries. *Transactions of the Royal Society of South Africa*, 51: 115-137.

Wong, S.C., Xiangdong, L., Thornton, I. (2006). Urban environmental geochemistry of trace metals. *Environmental Pollution*, 142: 1-16.

APPENDIX

Table A. 1 - Abiotic parameters (average ± SD) obtained for each site and season.

Site	Season	Depth (m)	Turbidity (m)	Temperature (°C)	Salinity (PSU)	pH	O_2 (mg/L)
ARE	**Summer**	1.19 ± 0.35	0.58 ± 0.12	21.53 ± 2.12	17.72 ± 6.05	8.28 ± 0.37	9.75 ± 3.10
	Autumn	1.22 ± 0.46	0.57 ± 0.21	16.26 ± 3.00	8.67 ± 2.10	7.71 ± 0.29	8.41 ± 2.36
	Winter	1.23 ± 0.34	0.47 ± 0.09	12.46 ± 2.88	7.13 ± 4.26	7.96 ± 0.18	8.71 ± 0.77
	Spring	1.19 ± 0.27	0.59 ± 0.19	21.28 ± 0.77	10.00 ± 1.12	8.30 ± 0.26	8.51 ± 0.79
BAR	**Summer**	2.12 ± 0.94	0.95 ± 0.20	19.10 ± 1.63	33.90 ± 2.33	8.16 ± 0.25	8.35 ± 1.90
	Autumn	2.43 ± 0.90	0.95 ± 0.38	16.32 ± 2.45	32.82 ± 2.34	8.09 ± 0.66	7.28 ± 2.06
	Winter	2.27 ± 1.03	0.87 ± 0.30	13.12 ± 2.20	28.07 ± 5.84	8.28 ± 0.57	8.09 ± 0.92
	Spring	3.30 ± 2.29	1.14 ± 0.33	17.03 ± 1.09	32.99 ± 2.75	8.18 ± 0.16	8.04 ± 1.57
CAR	**Summer**	2.57 ± 0.70	0.46 ± 0.12	22.52 ± 1.03	32.94 ± 3.30	7.93 ± 0.51	8.93 ± 3.09
	Autumn	2.63 ± 0.44	0.49 ± 0.06	17.31 ± 4.69	25.08 ± 4.08	8.28 ± 0.72	7.59 ± 2.16
	Winter	3.01 ± 0.62	0.70 ± 0.25	12.61 ± 2.44	19.07 ± 7.18	7.96 ± 0.54	7.63 ± 0.96
	Spring	3.01 ± 0.34	0.50 ± 0.09	18.98 ± 1.29	28.44 ± 2.01	7.72 ± 0.10	6.62 ± 1.60
GAF	**Summer**	3.11 ± 2.08	0.76 ± 0.37	19.09 ± 1.28	35.35 ± 1.49	7.94 ± 0.17	7.49 ± 1.99
	Autumn	3.49 ± 1.91	0.77 ± 0.20	15.85 ± 2.82	31.21 ± 3.62	8.23 ± 0.46	7.01 ± 1.61
	Winter	3.26 ± 2.59	1.00 ± 0.76	12.58 ± 1.97	24.60 ± 8.11	8.16 ± 0.35	8.18 ± 0.61
	Spring	3.85 ± 2.22	0.73 ± 0.17	17.88 ± 1.30	31.98 ± 3.52	7.97 ± 0.17	6.55 ± 0.79
LAR	**Summer**	2.40 ± 1.04	0.62 ± 0.24	21.66 ± 1.62	34.43 ± 2.37	7.99 ± 0.46	7.83 ± 2.11
	Autumn	1.90 ± 0.62	0.69 ± 0.24	16.91 ± 3.22	27.63 ± 6.68	7.79 ± 0.56	7.14 ± 1.68
	Winter	2.26 ± 0.92	0.61 ± 0.16	12.55 ± 2.81	17.89 ± 8.77	7.60 ± 0.34	7.75 ± 0.82
	Spring	2.53 ± 1.07	0.61 ± 0.14	18.88 ± 3.01	24.98 ± 5.17	7.79 ± 0.21	6.14 ± 0.88
POR	**Summer**	11.33 ± 2.08	1.83 ± 0.15	18.30 ± 1.08	34.20 ± 0.75	8.39 ± 0.74	6.36 ± 1.54
	Autumn	8.25 ± 0.35	1.05 ± 0.21	16.25 ± 1.20	31.10 ± 3.11	8.08 ± 0.01	9.65 ± 1.34
	Winter	7.65 ± 4.45	0.95 ± 0.35	13.80 ± 1.56	26.50 ± 0.99	8.10 ± 0.04	8.06 ± 0.62
	Spring	7.50 ± 0.50	1.30 ± 0.87	16.67 ± 1.81	31.20 ± 2.59	8.19 ± 0.02	8.39 ± 1.79
RIO	**Summer**	2.13 ± 2.07	0.88 ± 0.69	19.48 ± 1.55	34.69 ± 1.86	8.12 ± 0.45	7.72 ± 1.67
	Autumn	1.97 ± 1.68	0.81 ± 0.27	16.34 ± 3.25	26.86 ± 4.48	7.97 ± 0.44	7.06 ± 2.14
	Winter	2.00 ± 1.06	0.80 ± 0.20	12.06 ± 2.26	19.78 ± 8.10	7.82 ± 0.25	7.99 ± 0.67
	Spring	3.23 ± 2.46	0.70 ± 0.13	17.70 ± 2.03	25.66 ± 5.68	8.00 ± 0.15	6.65 ± 0.76
SJA	**Summer**	2.31 ± 1.13	0.92 ± 0.42	18.00 ± 1.69	35.60 ± 1.04	8.16 ± 0.26	8.43 ± 1.75
	Autumn	2.55 ± 0.84	1.01 ± 0.59	16.24 ± 2.99	34.51 ± 1.30	8.05 ± 0.42	8.11 ± 1.99
	Winter	2.59 ± 0.88	0.76 ± 0.15	12.74 ± 2.27	31.28 ± 5.36	7.98 ± 0.22	8.30 ± 1.09
	Spring	2.66 ± 1.42	1.16 ± 0.68	16.28 ± 1.14	35.22 ± 1.09	8.11 ± 0.22	8.18 ± 1.33
TOR	**Summer**	2.18 ± 1.72	0.90 ± 0.89	20.27 ± 1.53	34.40 ± 1.43	8.21 ± 0.47	8.76 ± 1.80
	Autumn	2.18 ± 1.40	0.67 ± 0.21	16.76 ± 3.77	27.66 ± 4.89	8.18 ± 0.56	7.79 ± 1.43
	Winter	2.46 ± 1.69	067 ± 0.23	12.94 ± 1.76	23.71 ± 6.84	8.39 ± 074	8.12 ± 0.86
	Spring	3.12 ± 1.92	0.67 ± 0.20	18.18 ± 1.61	30.45 ± 3.26	8.07 ± 0.19	8.17 ± 1.91
VAG	**Summer**	2.74 ± 0.81	0.51 ± 0.10	20.33 ± 2.10	29.61 ± 5.23	7.59 ± 0.48	6.35 ± 1.64
	Autumn	2.60 ± 0.80	0.77 ± 0.45	14.89 ± 4.22	20.46 ± 5.04	7.51 ± 1.00	6.66 ± 2.44
	Winter	2.87 ± 0.58	0.80 ± 0.71	11.57 ± 3.07	9.80 ± 8.50	7.52 ± 0.53	8.04 ± 0.55
	Spring	2.39 ± 0.56	0.51 ± 0.04	20.73 ± 1.65	22.67 ± 2.12	7.39 ± 0.16	5.05 ± 0.39

Table A. 2- Maturation scale for *Sepia officinalis* (adapted from Alonso-Allende and Guerra, 1984).

Maturation Stage	**Males**	**Females**
Stage I - Immature	Genital organs only perceptible	Small and white-yellowed ovary. Nidamental glands only perceptible. Yellowed and small (d<2mm) oocytes
Stage II - Developing	Undeveloped genital tract, small testicle. Needham' sac only visible.	Small and medium size yellowed ovary. Reduced nidamental glands, namely accessory ones, white and grey colour. Oocytes with d< 2mm and higher dimension reticulated and whitened oocytes (2mm<d<4mm)
Stage III – Maturing	Well-developed genital tract, medium size testicle visible. Small Needham's sac with no spermatophores.	Big size yellowed ovary. Presence of a jelly green coloured mass on the rear side of the ovary. Well-developed accessory nidamental glands of beige, yellow or orange colour. Small oocytes with d< 2mm. Medium oocytes (2mm<d<4mm) ad big yellow rounded reticulated oocytes (4mm<d<6mm).
Stage IV - Ripe	Big Testicle and Needham' sac with spermatophores, sometimes grabbed to the mantle walls or other organs.	Small and medium size yellowed ovary. Reduced nidamental glands, namely accessory ones, white and grey colour. Oocytes with d< 2mm and higher dimension reticulated and whitened oocytes (2mm<d<4mm)
Stage V – Post-Posture	Flaccid testicle. spermatophores are only present on the Needham's sac.	Small white-yellowed ovary, in a flaccid bag, mostly empty. Flaccid nidamental glands. Fewer oocytes in the ovaries