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ACCUMULATION, RESPONSES AND GENOTOXICITY OF TRACE ELEMENTS IN *OCTOPUS VULGARIS*

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Dedico este trabalho a duas mulheres fantásticas:

A minha Mãe e a minha Avó

"Quando o "porquê" é forte, o "como" torna-se fácil"
Lou Radja

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Sumário

Polvo, *Octopus vulgaris*, é um cefalópode sedentário que habita a zona costeira susceptível a estar exposto à contaminação. Foram capturados polvos em três locais da costa portuguesa com diferente contaminação: Matosinhos, Cascais e Olhão. Os teores de V, Cr, Fe, Co, Ni, Zn, Cu, As, Se, Cd, Hg e Pb foram determinados em glândula digestiva, brânquias, rim, gónadas, glândulas salivares posteriores, corações branquiais, saco de tinta, estômago, pele, manto e braços. A diferença nos níveis de metais entre os 11 tecidos analisados no polvo está aparentemente associada ao papel destes elementos nas funções metabólicas, estando os metais não-essenciais associados a ligandos específicos ou a mecanismos de desintoxicação. Os níveis de metais nos tecidos do polvo variaram com as concentrações registadas no ambiente. A análise das razões isotópicas de Pb na glândula digestiva permitiu uma separação, em função das fontes de Pb (antrópicas e naturais), entre os organismos capturados em Matosinhos e Olhão. Os níveis de Hg também variaram com a disponibilidade ambiental e, pela primeira vez, os teores de MeHg foram determinados no polvo. A glândula digestiva apresentou as concentrações mais elevadas de Hg, Se e MeHg, sendo a sua percentagem superior no manto. As boas correlações de Hg e MeHg entre a glândula digestiva e o manto sugerem um transporte eficiente da glândula digestiva para o manto. O Se parece ter um papel importante na protecção contra a toxicidade do Hg. Os processos de desmetilação parecem ser mais pronunciados nos organismos capturados na área com maior contaminação (Olhão). Só uma pequena percentagem dos metais acumulados fica associada aos organelos. Contudo, os níveis nestas fracções respondem aos incrementos observados no tecido. Os resultados obtidos sugerem que o papel destes elementos nas células, e consequentemente a associação com as fracções sub-celulares, é mais importante que a disponibilidade dos mesmos. Dentro das fracções citosólicas, os metais encontram-se associados a proteínas de elevado e baixo peso molecular. As metalotioninas apresentam-se como um mecanismo de desintoxicação (e.g. Cd) apenas quando os níveis excedem um valor limite. Apesar dos diferentes mecanismos de desintoxicações, foram registados danos ao nível do DNA, principalmente na glândula digestiva do polvo capturado na área com maiores níveis de Cd. Foi observado um paralelismo com a disponibilidade ambiental, a função do tecido e a renovação celular. Estes resultados sugerem que o polvo é um potencial bioindicador de contaminação.

Summary

Common octopus, *Octopus vulgaris*, is a sedentary cephalopod inhabiting coastal waters and thus susceptible to be exposed to local contamination. Octopuses were captured in three coastal areas with contrasting environmental contamination: Matosinhos, Cascais and Olhão. Levels of V, Cr, Fe, Co, Ni, Zn, Cu, As, Se, Cd, Hg and Pb were analysed in various tissues of octopus: digestive gland, gills, kidney, gonads, posterior salivary glands, branchial hearts, ink sac, stomach, skin, mantle and arm. The different metal concentrations in the eleven analysed tissues are apparently a consequence of the role of metals in metabolic functions (e.g. gonads, ink sac, kidney, gills and salivary glands), although non-essential elements in digestive gland, branchial hearths, kidney and ink sac may be linked to specific ligands or excretory/detoxification mechanisms. Metal levels found in octopus tissues (e.g. Cd, Pb and Hg) were in line with concentrations registered in the environment. Lead isotopic ratios in octopus digestive gland allowed separation of individuals according to environmental Pb sources (anthropogenic and natural). The consistent differences between organisms captured in the two areas (Matosinhos and Olhão) points that Pb isotopic signature provides a useful tool to distinguish octopus populations. Concentrations of Hg also responded to environmental availability and, for the first time, levels of MeHg were determined. Higher Hg, MeHg and Se concentrations were observed in digestive gland and MeHg (%) in mantle. Good relations were obtained between digestive gland and mantle for Hg and MeHg, suggesting an efficient transport from digestive gland and storage in mantle. Selenium seems to have a protective role against Hg assimilation. Demethylation processes may occur being more noticeable in organisms from the more contaminated area. When metals are accumulated, only a minor percentage is associated with organelles. However, levels in these sub-cellular fractions respond to the enhanced concentrations in the whole tissue. Moreover, it seems that the role of the elements in the cells, and consequently their association with the sub-cellular fraction, superimpose the response to availability. Within cytosolic fraction metals are associated either with low and high molecular weight proteins, being metallothioneins an important detoxification mechanism when levels, mainly Cd, exceeded a threshold value. Although different detoxification mechanisms were observed in octopus tissues, DNA damages were registered mainly in digestive gland. A good agreement was obtained with environmental availability, tissue function and cell-turnover. Cadmium seems to be a strong strand breakage inducer. Octopus can be used as bioindicator.

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Chapter 1

General introduction

Context

This chapter presents an overview of the issues developed in this thesis. The first part reviews the accumulation pathways and detoxification mechanisms found in organisms. It also briefly describes the processes leading to sub-lethal effects, e.g. genotoxic effects. The second part describes the octopus biology and contamination, presents the study sites and the main objectives of this thesis.

Introduction

Coastal environment

From an environmental point of view, the coastal environment is a geographic space influenced by terrestrial inputs and intense processes of bio-production (Fernandes, 1997; Castro et al., 1999). Sixty percent of the existing large cities, wich comprise more than 2.5 million people, are located near the coast. A substantial proportion of wastewater generated from human activity reaches directly or through rivers the coastal environment with little or no treatment (Islam and Tanaka, 2004). Concerns of long-term adverse effects of contaminants on aquatic ecosystems emerged in the last decades (van der Oost et al., 2003). In addition, the fate and effects on exposed target organisms have also been extensively studied in the aquatic ecosystem (e.g. Depledge and Fossi, 1994). In line with the potential risks for the coastal ecosystem wellbeing the European Water Framework Directive (WFD; 2000/60/EC) establishes a framework for the protection and improvement of ecological quality in transitional and coastal waters. The aim is to achieve a good quality water status for all aquatic systems (Muxika et al., 2007). In particular, the efforts on restoring impacted ecosystems have been widely welcomed by scientists and environmental managers (Kowalski, 2009, references herein). However, coastal environments are dynamic and complex ecosystems, and spatial-temporal variability associated from natural processes may mask the effect of anthropogenic pressures.

Trace elements in marine organisms

Trace elements are widely found in marine organisms, reflecting its availability on the environment. Some trace elements at certain concentration intervals are important for organisms, playing an essential role in tissue metabolism and growth (Leland and Kuwabara, 1985). For example, Cu, Zn and Fe are known as vital components of enzymes, respiratory proteins and certain structural elements of organisms (Depledge and Rainbow, 1990). Manganese, Se and Co have also important roles in various cellular components like, pyrovate carboxylase, glutathione peroxidase and vitamin B12, respectively. While a range of trace metals must be delivered to the tissues of an organism in order to meet the diverse metabolic requirements, accumulation of potentially toxic metal species may also occur. Indeed, some elements display high concentrations in tissues of marine organisms, and the question is whether it results from natural processes or influenced by the increasing availability in contaminated areas. Metals, such as Cd, Hg and Pb, have been considered as non-essential because they have no known biological role; these metals become highly toxic when found associated with metabolically active sites, even at relatively low concentrations (Rainbow, 1985).

Assimilation pathways. The uptake of trace elements by marine organisms can occur from water, including suspended particulate matter, food and sediments. The exposure of organisms to contaminants via food and water will depend on the ecological lifestyles of the aquatic organisms (Valavanidis et al., 2006). Uptake via water can take place across the whole body surface, in addition to the gills, in

organisms lacking external shells (e.g. cephalopods) and in all molluscs some absorption of dissolved metal across the digestive epithelium (Langston et al., 1998). Nevertheless, since the branchial epithelium represents the primary target for waterborne due to their respiratory/nutritional functions, enhanced metal levels are often found (Langston et al., 1998; Pan and Zhang, 2006). Food may also be a significant source of metals, if not the primary source, to organisms. Studies suggested that metal partitioning in tissues such as digestive gland and muscle, are mostly affected by metals accumulated from food, while for gills the major vector is water (e.g. Langston et al., 1998). Water-soluble (hydrophilic) elements are more bioavailable to organisms than water-insoluble (hydrophobic) elements that are strongly sorbed or bound to suspended particles, dissolved organic matter, or biological systems (Rand and Petrocelli, 1985). Class B elements such as Cu, Zn and Cd, form a wide range of covalent compounds and are therefore less likely to exist as free ions in solution (Simkiss and Mason, 1984). Usually they are present in biological tissues as divalent cations, which are free or complexed to different classes of biological ligands (Soto and Marigómez, 1995). These elements can be bound to sulphydryl, hydroxyl, carboxyl, amino residues of proteins, peptides, aminoacids at the amino (–NH) and carbonyl (–C=O) groups of the protein chain backbone (Soto and Marigómez, 1995). Nevertheless, due to the difference in atomic number and electronegativity, affinity for the different class of ligands may vary in a great deal (Rainbow, 1993). Due to this broad affinity, many different uptake processes may be involved and the rate at which the metal enters the organism is related to the level in the environment (Simkiss et al., 1982).

Bioaccumulation. Bioaccumulation in a tissue is the net balance between uptake and depuration rates of an element (Brown and Depledge, 1998). The different accumulation strategies go from a strong accumulation and weak depuration to weak accumulation and strong 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). Essential metals may be subject to regulation either by limiting metal uptake at the level of the body concentrations or by involving organism-specific accumulation strategies with active excretion from the metal excess pool and/or storage in an inert form and/or excretion of stored (detoxified) metal (Figure 1). Whereas for non-essential metals, excretion from the metal excess pool and internal storage without elimination are the major strategies (Rainbow, 2002). Aquatic organisms take up and accumulate trace metals whether essential or not, all of which have the potential to cause toxic effects (Rainbow, 2007). Uptake of non-essential elements is almost universally determined by the degree of exposure. In contrast, body burdens of some essential metals may be less influenced by external concentrations, suggesting various degrees of homeostasis (Langston et al., 1998).

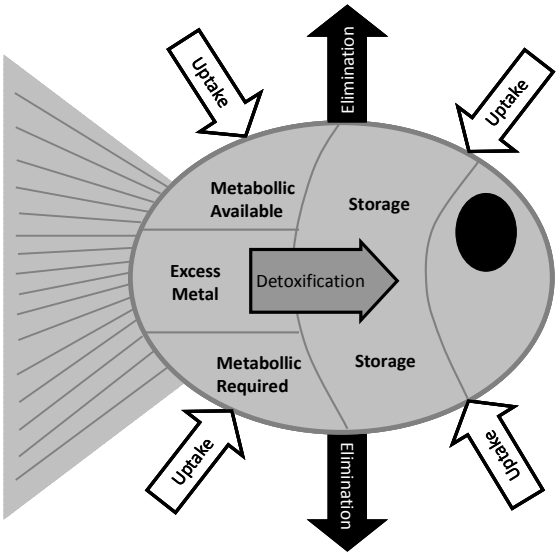

Figure 1.1 – Mechanisms occurring in organisms: uptake, storage and detoxification of contaminants.

Detoxification of accumulated trace elements

Whether the well-being of the organism is eventually affected by the presence of trace elements at undesirable concentrations may depend upon many factors, some intrinsic (e.g. age, sex, health and nutritional status of the organism) and others extrinsic (e.g. dose, duration, route of exposure to the contaminant and the presence of other chemicals) (van der Oost et al., 2003). Organisms may survive within environments containing toxic chemicals in spite of the tendency to overload the normal physiological mechanisms of biotransformation or detoxification present in the cell (Moore, 1985). Metals in excess are potentially toxic and should be removed from the vicinity of important biological molecules to maintain the regular function of cells (Vijver et al., 2004).

The tolerance of marine organisms is associated with the presence of detoxifying mechanisms to prevent toxic substances from affecting metabolism or damaging sensitive structures within cells. Sub-cellular 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). Detoxification mechanisms include: redox reactions of metals enhancing elimination; once in the cytoplasm, interaction of metals with high-molecular-weight ligands (HMW, such as metalloenzymes), low-molecular-weight ligands (LMW, e.g. glutathione) and metallothioneins (MTs) (Di Giulio et al., 1995), and sequestration of toxicants in less mobile tissues, or organelles, thus limiting the access to the more sensitive tissues and organelles (Leland and Kuwabara, 1985). Overall, systems at sub-cellular level may be activated in order to prevent deleterious effects. The onset of toxicity can occur 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).

***Metallothioneins*.** Metallothioneins (MTs) were firstly isolated from equine renal cortex by Margoshed and Vallee (1957). They are cytosolic proteins characterised by low-molecular-weight (6-7 KDa, 57-75 amino acids), high thiol richness (18-20 cysteines per molecule), heat stability, and lack of aromatic amino acids (Viarengo, 1989; Viarengo and Nott, 1993; Simes et al., 2003; Vergani et al., 2007 and references herein). Due to the sulphur atoms of cysteine residues, MTs are able to bind very strongly and specifically some class B elements such as Cu, Cd, and Zn, forming metal-thiolate complexes (Dallinger, 1995). MTs are now though to be almost ubiquitous among aquatic organisms being reported for some 50 different aquatic invertebrates (Langston et al., 1998). They are most abundant in parenchymatous tissues (i.e., liver, kidney, pancreas and intestines) but their occurrence and biosynthesis have been document in many tissues and cell types (Pourang et al., 2004). The most important functions of MTs are: the essential element homeostasis (e.g. Cu and Zn); metal detoxification by way of induction (e.g. Cd and Hg); radical scavenging; and gene regulation (Thornalley and Vasak, 1985; Roesijadi, 1992, 1996; Dallinger, 1995; Langston et al., 1998; Park et al., 2001). It seems clear that most of the MTs functions are related to cellular stress events. What makes these proteins so significant in the cell is the fact that they may meet different demands simultaneously. While MTs detoxify excess amounts of Cd, for instance, at the same time, they have to supply cellular compartments with essential elements (Dalinger, 1995) by donating Cu and Zn to appropriate receptor molecules (metalloenzymes, respiratory pigments, nuclei acids and membranes).

Various studies have showed that MTs, in marine invertebrates, are employed as a detoxification strategy (Bebianno and Langston, 1991; Roesijadi, 1992; Viarengo and Nott, 1993). Experimental works have proved that trace elements can act, at certain levels, as effective MTs inducers (Bebianno et al., 1993; Bebianno and Serafim, 1998; Lueng and Furness, 2001; Ng and Wang, 2004; Shi and Wang, 2005). The MTs production has also been recorded in organisms exposed to complex mixtures of contaminants under environmental conditions (Geffard et al., 2002; Bebianno and Serafim, 2003; Smaoui-Damak et al., 2004). Important to the assessment of the degree of toxicity is to determine the amount of metal that is bound to MTs. The fact that organisms have the capacity to synthesize these metalloproteins that can sequester and subsequently detoxify metals implies that an increased body burden of metals will not necessarily result in increased toxic effects (Di Giulio et al., 1995). Inducible metal-binding proteins may provide an initial high-affinity mechanism for control of metals within the cell, since protein turnover is relatively rapid (Fowler, 1987).

***Organelles*.** Organelles are probably a stable compartment to storage toxic elements. The cell is deemed to be the most basic structural and functional unit of all living organisms and is often called a “building block of life” (Chou and Shen, 2007). Organelles, one of the constituents of the cell, are specialized to carry out different tasks. They are recognised to be sensitive to metal contamination and its examination may provide a better understanding of potential mechanisms of toxicity and tolerance (Roesijadi, 1981;

Wallace et al., 2003). The partition of metals in these sub-cellular fractions is related to the fact that storage takes place in compartments that are particularly rich in, or capable of synthesizing relatively large quantities of metal-binding ligands (Langston and Spence, 1995). The impacts on trophic transfer of metals may be evaluated according to the fraction where metals are accumulated. Metals associated with lysosomes, mitochondria, HSP and HDP fractions may be trophically available for transfer to predators, whereas metals associated with granules and cellular debris may be unavailable for transfer (Wallace et al., 2003).

Within cells there is an intricate network of organelles that all have unique functions (Figure 2). These organelles allow the cell to function properly. The nucleus of the eukaryotic cell contains the genetic material (DNA) governing all functions of the cell (Chou and Shen, 2007). Granules are fairly ubiquitous in molluscs, though they may serve different functions within distinct cells in relation to the distribution of metals (Langston et al., 1998). Mitochondria are multifunctional cellular organelles with both energetic and ion-sequestration functions (Chavez-Rooker et al., 2002; Chou and Shen, 2007). It is considered as a more metal-sensitive compartment (Bonneris et al., 2005), because metals can bind to crucial enzymes and respiratory protein complexes. Accumulated metals in the mitochondria fraction reduce energy conversion efficiency and uncouple oxidative phosphorylation that causes oxidative damage (Di Giulio et al., 1995). Lysosomes are membrane-bound cell organelles containing hydrolytic enzymes and involved in intracellular digestion (Cajaraville et al., 1995). They play a role in the normal turnover of cytosolic proteins such as MTs, providing means for metal accumulation in the internal lysosome milieu (Fowler, 1987; Dallinger, 1995). They are known to be involved in sequestration functions reducing the negative effect of high accumulated metal concentrations in other organelles, metals are precipitated within the lysosome and complexed (Viarengo et al., 1985, 1987). The functional consequences of metal accumulation in these cellular structures may result in the inhibition of enzyme activities, disruption of the normal process of lysosomal biogenesis causing functional impairment of this essential cellular system (Fowler, 1987). Another sub-cellular fraction that should be considered is the microsome, a vesicle-like artifacts formed from the endoplasmic reticulum after cells broke-up during centrifugation. It has been proposed that metals in this fraction could point to toxicity due to the presence of fragmented endoplasmic reticulum, which is generally responsible for synthesis and transport of proteins (Jarosch et al., 2002; Bonneris et al., 2005; Chou and Shen, 2007).

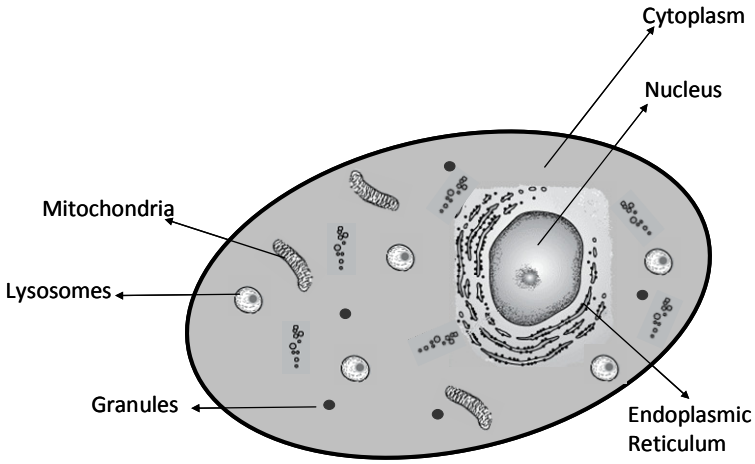

Figure 1.2 – Schematic example of a eukaryotic cell, with the various components/organelles.

Sub-lethal effects

It is documented that a wide range of cellular activities are involved in the response of organisms to environmental metals. Once metals entered the cells they undoubtedly become bound to a variety of ligands and it is the metabolism of these complexes that determine the subsequent fate of metals and the final body load (Simkiss and Mason, 1984). However, due to surpass of the capacity of detoxification systems to protect the cell, damages may occur.

***Genotoxicity*.** Among the molecular components of the cell, DNA is an important target of environmental stress in organisms (Frenzilli et al., 2001). DNA is present in the cell as a functionally stable, double-stranded polymer without discontinuity (strand breaks) or abnormal modifications and is complexed with proteins (Shugart, 1995). The exposure of organisms to metal contamination promotes interactions between metals and DNA (Figure 3). The interactions are manifested primarily by structural alterations in the DNA molecule and can take the form of adducts (where the chemical or it metabolite becomes covalently attached to the DNA), of mutations, of strand breakage, or of chemically altered bases (Shugart, 1995; 2000) and eventually carcinogenesis and other health disorders (Kurelec, 1993). DNA is the only molecule with capacity for self-repair (Shugart, 1995). However, the ability to repair depends on exposure. If a DNA lesion induced by a metal can be repaired before fixation, there may be no effect on DNA. However, this is only true in low-levels exposure where repair enzymes are not saturated by significant numbers of damaged DNA sites (Shugart, 1995). Because all organisms exhibit this response, the increased environment contamination leads to an enhancement in the levels of repair indicating DNA toxicity. Changes in the integrity of DNA have been proposed as useful endpoints for assessing the effects of environmental pollutants at individual, population and ecosystem level (Klobucar et al., 2003). The single–cell gel electrophoresis (Comet) assay, has become a widespread technique for detection of DNA damage induced by xenobiotics (e.g. Cd, by Desai et al., 2006; Fourie et al., 2007; Hg, by Tran et al., 2007; organic compounds, by Costa et al., 2008). The alkaline version of the assay has proven to be a simple and

reliable method for the quantitation of total DNA fragmentation as a result of the formation of single– and double–strand breakage, xenobiotic–DNA adducts and alkali–labile sites (e.g. unstable altered nucleotides) (Singh et al., 1988). Nevertheless, the mechanistic of genotoxicity is still poorly known and thus the relative potency of contaminants to induce DNA damage and the differential susceptibility of various organs towards genotoxic damage still need further research. Moreover, the majority of studies deal with one and/or a limited number or combinations of contaminants, and thus research in aquatic ecosystems with complex mixtures and interactions of metals and other contaminants is still missing.

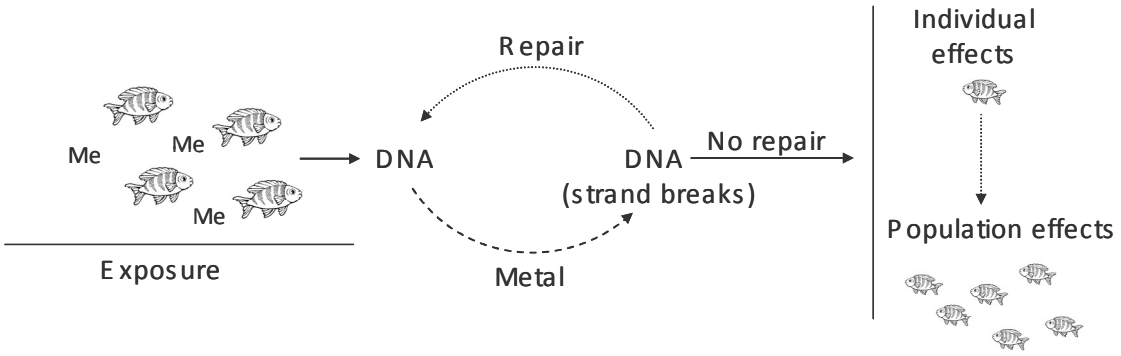

Figure 1.3 – Schematic representation of processes leading to DNA damages from exposure to effects on populations (Me – metals).

***Octopus vulgaris* (common octopus)**

General characteristics. *Octopus vulgaris* belongs to the class Cephalopoda (Fisher et al., 1987; Mangold, 1983), which is considered as the most active, intelligent and specialized class of molluscs. Octopus have a soft-bodied that consists of: a muscular mantle that houses the internal organs and represents 8% of body weight in adults (Trueman and Packard, 1968); and 8 circumoral arms (no tentacles) with bases connected by a membranous web, and suckers in two series, without chitinous rings or hooks, along the length of the arms (Jereb et al., 2005). The arms account for 70% of its body weight (Dilly et al., 1964). The mouth has a pair of chitinous jaws (the beaks) and, as in other molluscs, a chitinous tongue-like radula (band of teeth) (Jereb et al., 2005). They lack any internal shell which allowed the development of the powerful muscular mantle that became the main locomotory organ for fast swimming, via water jetting from the funnel (Jereb et al., 2005). The circulation of water through the mantle not only produces the power for swimming, but it provides oxygen for their gills. The surface area of cephalopod gills have been much increased by a type of folding and are not ciliated as in other mollusks (Gonçalves, 1993). These cilia are unnecessary since cephalopods are predators not filter feeders. The circulation of water over the gills is the reverse of what it is in the gastropods, since water leaves the mantle cavity by means of the funnel (Wells, 1978). Also, the digestive system works with the circulatory system to provide the nutrients needed to keep the heart pumping. Octopus have a closed circulatory system, with a principal, or systemic, heart, two branchial hearts and developed arterial, venous and capillary systems supplying

blood to the muscles and organs (Jereb et al., 2005). The nervous system is highly developed, with a large brain and peripheral connections. Octopus has the most complex brain of all the invertebrates, just like vertebrates, they have long term and short-term memories. They are able to change color by using a complex system of chromatophores under nervous control. The chromatophores are pigment-filled sacs present in the skin, and capable of remarkable expansion and contraction. This system responds to current situations in the environment. They produce ink, a dark, viscous fluid also expelled through the funnel. The ink may take the form of a mucoidal 'pseudomorph' (false body) to decoy potential predators, or of a cloud to obscure the escaping cephalopod. The common octopus is a benthic, neritic species occurring from the coast line to the outer edge of the continental shelf, in depths from 0-200 m, where it is found in a variety of habitats (Vaz-Pires et al., 2004). It is normally solitary and territorial, using cavities or digging a burrow as a home for itself, and leaves it only to feed or reproduce. They often protect and hide their homes with shells (called the *middens*), stones and other solid things that they gather. Both sexes are randomly distributed within patches of different density and are also randomly spaced (Mangold, 1983). When not travelling in- or offshore, *O. vulgaris* seems to be a truly sedentary species. Octopus is a predator being exploratory and opportunistic (Mather and O'Dor, 1991). They feed mainly on crustaceans, fish and bivalves, leaving the dens at dusk to go for hunting trips, and return at dawn (Wells, 1978). Preys are killed by means of a secretion produced in the posterior salivary glands, cephalotoxin (Wells, 1978). Octopuses have fast growth rates, up to 13% body weight per day, and food conversion rates 15-43% (Mangold, 1983; Navarro and Villanueva, 2003). They have been long considered of cosmopolitan occurrence in temperate and tropical seas (Roper et al., 1984), although a possible occurrence of cryptic species among *O. vulgaris*-like octopods is also reported (Guerra et al., 1999). Thus, the distribution of *O. vulgaris* in a strict sense may be restricted to the Mediterranean Sea and eastern Atlantic Ocean (Mangold, 1983). Throughout its distribution range, this species is known to undertake limited seasonal migrations, usually overwintering in deeper waters and occurring in shallower waters during summer (Roper et al., 1984).

Birth and offspring. *O. vulgaris* have a short life span of 12 to 18 months. In the early spring, adult octopus move closer to the shore for spawning (Mangold and Boletzeky, 1973). They have separate sexes, and fertilization is internal. Within 2 months after mating, the female releases up to 500,000 eggs (Mangold, 1983). They are laid in shallow water and are always attached to a substrate, between rocks and coral reefs. On sandy or muddy bottom, eggs are laid in empty shells or in man-made objects such as cans, bottles or tires. The female take care of the eggs providing oxygen by squirting them with streams of water and cleans them with the suckers. She also defends them from predators until they hatch. Soon after the eggs have hatched the female dies. At hatching, this species has very small hatchlings, paralarvae (2 mm mantle length) (Boletzky, 1987). Paralarvae are planktonic for 1–3 months, depending on the effect of temperature on growth rate, and adopt the benthic life mode of the adults at around 7.5 mm ML (Villanueva, 1995). The change from planktonic to benthic life is not sudden but is a gradual process

(Boletzky, 1977), and it does not imply a total change in feeding behaviour. Growth is very rapid and juveniles can reach 0.5-0.6 kg within six months of hatching and 1.4-1.8 kg within eight months (Iglesias et al., 2004). Studies on the Portuguese shelf highlighted the role of temperature and upwelling in modulating seasonality and distribution of *O. vulgaris* paralarvae. The influence of the physical environment was especially pronounced for this specie (Moreno et al., 2009).

***Fisheries and Aquaculture*.** Cephalopods seem to be one of the remaining marine resources, in some areas, that still experience an increase in landings (Caddy and Rodhouse, 1998). In Portugal the landings and the economic value of cephalopods have, over the years, maintained a significant growth, indicating an increasing dependence of the fisheries economy on its landings (Pierce et al., 2010). A study by Campos et al. (2007) in the Portuguese trawl fleet showed that octopus accounted for a high proportion of the total landings of these vessels. It is currently ranked third in landings and generates the highest revenue of all species taken in Portuguese fisheries (Pereira et al., 1997). *O. vulgaris* is captured by various methods: hooks and lines, pots and traps in small-scale coastal fisheries in depths of 20-200 m (Pierce et al., 2010). It is marketed fresh, frozen and dried salted, mostly for human consumption. Despite their widespread consumption and high market value, commercial aquaculture of cephalopods is very recent and so far concerns this specie (Vaz-Pires et al., 2004). As mentioned above, octopus presents a series of characteristics favourable for commercial farming, including: very fast growth rate (Mangold, 1983; Miliou et al., 2005), high feed conversion efficiencies (Mangold and Boletzky, 1973), high reproductive rate (Iglesias et al., 1997), a great tolerance to captive conditions (Iglesias et al., 2000), and a high market price (Vaz-Pires et al., 2004). However, important problems remain regarding the culture of early life stages, planktonic paralarvae (Iglesias et al., 2007), restricting farming practices to the ongrowing of subadults, typically obtained from fisheries (Iglesias et al., 2000; Chapela et al., 2006; Rodríguez-Domínguez et al., 2006).

Cephalopods metal contamination

Cephalopods represent an essential link in marine trophic chains and are eaten by many top predators. They are known for their ability to accumulate high levels of essential and non-essential elements to metabolic functions (e.g., Martin and Flegal, 1975; Miramand and Guary, 1980; Mangold, 1983; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante et al., 1998a, b; Bustamante et al., 2000).

Influence of biological parameters. The effect of biological parameters (i.e., size, weight and gender) on the metal accumulation in cephalopods is far from being consensual. Some works reported similar concentrations in small and large individuals (Barghigiani et al., 1991; Bustamante et al., 1998a; Raimundo et al., 2004; Seixas et al., 2005a), others indicated correlation between accumulated levels, such as Hg, and size (Monteiro et al., 1992; Rossi et al., 1993; Pereira et al., 2009). Negative relationships were also observed between Cd concentrations and weight (Storelli and Marcotrigiano, 1999). A variety of

situations were reported with respect to gender: no differences in metal accumulation between males and females (Miramand and Bentley, 1992; Monteiro et al., 1992; Bustamante et al., 1998a; Barghigiani et al., 2000; Seixas et al., 2005a, b), lower levels in females (Rossi et al., 1993; Pierce et al., 2008) and higher levels in females, like Zn (Seixas et al., 2005a; Bustamante et al., 2006). Instead, it appears that bioaccumulation processes in cephalopod tissues are strongly influenced by metal availability in the environment (including the food web).

Metal partitioning in tissues. Some studies have evaluated the partition of metals in tissues of cephalopods (Miramand and Bentley, 1992; Nessim and Riad, 2003; Raimundo et al., 2004, 2005; Napoleão et al., 2005; Bustamante et al., 2008; Pereira et al., 2009). A general conclusion can be extracted from all studies: for most of the elements (As is one exception) digestive gland accumulates higher levels. These findings are related with the intrinsic capacity of this organ to store these elements, which suggests a major role in detoxification and assimilation processes (e.g., Bustamante et al., 2002; Bustamante et al., 2006a; Storelli et al., 2006). Cadmium is mainly accumulated in this tissue, reaching 98% of the total body burden in some species (e.g., Miramand and Guary, 1980; Bustamante, 1998). Additionally, cephalopods also have the ability to concentrate high metal levels in other tissues. The branchial hearts are known to accumulate high concentrations of Cu, Fe, Zn, Cd, Ni, V and Mo (Miramand and Bentley, 1992; Nessim and Riad, 2003; Napoleão et al., 2005). The enhanced levels of Fe in the branchial hearts may be due to the presence of adenochromes (Ghireti-Magaldi et al., 1958). Gills tend to concentrate high levels of Cu (Nessim and Riad, 2003) and kidney of Mn, Ni and Pb (Miramand and Bentley, 1992). The elevated concentrations of Cu found in the branchial hearts and gills are probably associated with the presence of the heamocyanin (respiratory pigment), in which Cu is one of the main components (Soldevilla, 1987; Villanueva and Bustamante, 2006; Craig and Overnell, 2003). Metals in branchial hearts and kidney are probably associated with storage and excretory functions and detoxification mechanisms of these tissues (Schipp and Hevert, 1978; Guary and Fowler, 1982; Rainbow and Phillips, 1993; Villanueva and Bustamante, 2006).

Geographical variations. Metal accumulation in cephalopods, mainly in digestive gland, can reflect their origin (Bustamante et al., 1998b, 2000; Nessim and Riad, 2003; Raimundo et al., 2004; Seixas et al., 2005a, b). In some cases geographical variations of metal availability can overcome the biological differences (Nessim and Riad, 2003). Contrasting geographic patterns were observed in digestive gland of specimens collected in the Portuguese coast, with higher levels of Zn, Pb, Cu and Hg in organisms collected in Southern areas, while Cd increased drastically in Northern areas (Raimundo et al., 2004). These patterns were in good relation with water surveys in the Portuguese waters (Caetano and Vale, 2003). Similar results were obtained for octopus in the same area by Napoleão et al. (2005). Another study with cephalopods, showed differences between the ones collected in the sub-Artic area (higher Cd levels) compared with cephalopods from lower latitudes such as along the French Atlantic coast (Bustamante et al., 1998b). The enhanced levels in sub-Artic area were supported by high Cd concentrations in top

vertebrate predators. As a result, cephalopods have been proposed as important vectors of metal transference to top predators (Bustamante et al., 1998a, b).

Detoxification. These high levels found in the digestive gland would be expected to be toxic unless efficient regulation and detoxification processes are available (Simkiss and Taylor, 1982; Phillips and Rainbow, 1989; Bustamante et al., 2002). Several studies aimed to assess the mechanisms responsible for such "absence" of toxicity. Interactions between essential (Zn and Cu) and non-essential elements (Cd) were assessed in the digestive gland of *S. officinalis* and *O. vulgaris*, and explained as a competition for ligands, and a possible detoxification mechanism (Raimundo et al., 2005; Pereira et al., 2009), since relationships were more pronounced in specimens captured in areas with enhanced levels of Cd and positively related with weight. Finger and Smith (1987) and Tanaka et al. (1983) searched for associations between metals and protein in the squids, *Nototodarus gouldi* and *Ommastrephes bartrami*. Proteins with low, intermediate and high molecular weight were pointed out as potential binding sites for trace metals, mainly Cu, to a lesser extent Cd and little Zn. Studies have also evaluated relations in wild specimens of cephalopods to investigate how they mange to tolerate such amounts of Cd looking for the involvement of MTs and the subcellular distribution, however, relationships to Cd concentrations have not been found (Bustamante et al., 2002; Bustamante et al., 2006). Instead, they proposed that an alternative mode of detoxification may be activated as Cd reached a threshold, being the lysosomal fraction involved in this "new" process. Less data is available for other elements (Bustamante et al., 2006).

Human consumption. Cephalopods are an important food resource being consumed in large quantities in several countries world wide (Amaratunga, 1983). In general, mantle and arm of octopus, which are the commercial items contain low Cd concentrations, generally below the safety limit established by the European Commission (1.0 μg g^{-1}, ww of Cd, Journal of EU Communities 2001, EC rule no. 466/2001). More than 95% of octopus sampled in the Portuguese coast (Raimundo et al., 2005; Raimundo et al., 2009) presented levels below that limit. According to the joint FAO/WHO expert committee the Provisional Tolerable Weekly Intake (PTWI) recommended for Cd is approximately 7 μg Kg^{-1} body weight (WHO, 2003). On the basis of values registered in the two contrasting areas in Portuguese coast and assuming an average weight of 60 Kg for humans, the estimated values for PTWI ranged between 2 and 8 kg. Estimated values exceeds largely the weekly average consumption of fishery products in Portugal of 1120 g (FAO, 2007) pointing to a marginal or no risk of its consumption (Raimundo et al., 2009).

Portuguese Coast as study area

The Portuguese coast is extensive with 943 km and it is under pressure, as result of a fast growing development intensified since the mid 50's of the 20th century (Alves et al., 2007).

Morphologic features and oceanographic conditions. In the northern west coast, several rivers discharge directly into the sea, like the Minho, Douro and Mondego rivers. The river discharges have a pronounced seasonal variability, being higher in the winter (http://www.inag.pt) and influencing the stratification of the coastal waters (Moita, 2001). The Douro estuary, which is the end-member of the largest watershed of the Iberian Peninsula, is located in one of the most populated zone of Portugal and is subject to progressive human intervention. The Douro river plume is integrated into larger low salinity waters fed by the winter-intensified runoff of several rivers on the northwest coast of Portugal and Spain (Peliz et al., 2002; Alvarez et al., 2006). The plume is detected in a narrow band less than 20 km wide and its extension is about 100 km (Peliz et al., 2002; Santos et al., 2004). In the south and southwest coasts, major rivers, like Tagus and Sado are characterised by having large estuaries that tends to trap a great part of the material transported by low and moderate flows. Tidal currents cause daily suspension of topmost sediment layers and associated contaminants, and redistribution inside the estuaries according the morphology (Vale and Sundby, 1987). These systems may receive episodically abrupt quantities of freshwater and land-derived contaminants (Vale et al., 1990; Martins et al., 2005). In addition, the Portuguese continental shelf includes several canyons that influence water circulation (Fiuza, 1983).

Water currents and upwelling. Surface waters of the Iberian coast change circulation according to the season (Haynes and Barton, 1990; Pingree, 1993; Wooster et al., 1976), being, in winter, northwards to the Bay of Biscay in France, and in summer, it becomes weaker and reverses due to the N trade wind regime (Fiuza, 1983). This southward current promotes cooling and wind-induced upwelling along the shelf break (Fiuza, 1983; Abrantes and Moita, 1999). North of the Nazaré canyon, the coastal waters are characterised by a homogeneous upwelling of NACW along the shore (Fiuza, 1983). From Lisbon to Cape Sines, the upwelling is affected by the presence of coastal protrusions like Cascais, Lisbon and Setúbal canyons. To South of Cape Sines until Cape S. Vicente, the upwelling structure becomes more regular but is affected by warmer and saltier offshore surface waters (Moita et al., 2003).

Metals in Portuguese coastal waters. Cotté-Krief et al. (2000) analysed Cd, Cu, Ni and Zn in water off the continental Portuguese shelf and compared the influence of contaminated rivers (Tinto, Odiel and Tagus) and the coastal upwelling in defining levels in coastal waters. It was found that upwelling, contrarily to other systems in non-contaminated areas do not act as a source of trace metal enrichment of the continental margin waters. Concentrations of dissolved metals reported in that work were lower than values close to the shoreline in NW of Portugal (Leal et al., 1997). Surface water collected at 1 mile from the mouth of major estuarine systems in Portugal, pointed to the export of Cd and Cu from the rivers in the NW coast (Caetano and Vale, 2003).

Aims and Structure of the Thesis

The current work was developed with octopus samples from three coastal areas, Matosinhos (NW), Cascais (W) and Olhão (SE). These areas were selected according to previous studies on metal availability in water (Cotté-Krief et al., 2000; Caetano and Vale, 2003) and levels in octopus tissues (Raimundo et al., 2004; Napoleão et al., 2005; Seixas et al., 2005a, b). The contrasting metal values found in octopus tissues from these areas would constitute a good "*in situ* test" to test metal accumulation and evaluate responses and effects in different tissues of feral octopus. The aims of this Thesis are:

- To evaluate whether octopus exposed naturally to different trace elements availability display differences on accumulation and partition of metals and metalloids among tissues;
- To identify sub-cellular responses of octopus tissues to accumulated metals and metalloids;
- To estimate the effects of metal accumulation on DNA.

This thesis is composed by five Chapters, encompassing articles submitted or published at peer-reviewed journals:

Chapter 1 – General introduction

Chapter 2 – Elemental concentrations and tissue partitioning

Chapter 3 - Sub-cellular responses to elemental concentrations

Chapter 4 - Genotoxic effects

Chapter 5 – General Discussion

Appendix - Methodologies

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Chapter 2

Elemental concentrations and tissue partitioning

Context

As described in chapter 1, metal levels in tissues may provide an important tool to predict some possible responses and effects at an individual level. The identification of a target tissue and its role in the storage and detoxification processes is of extremely importance. Furthermore, it can permit the identification of new bioindicators species in monitoring programs.

Summary

This chapter describes the Fe, Zn, Cu, Cd and Pb levels in digestive gland, posterior salivary glands, kidneys, gills, gonads, branchial hearts, ink sac, stomach, skin, mantle and arm of octopus. It also presents the response of octopus digestive gland to differences in Pb levels and its stables isotopes in the environment, as well as, Hg and MeHg in digestive gland and mantle.

Chapter 2.1

Partitioning of Fe, Cu, Zn, Cd and Pb concentrations among eleven tissues of *Octopus vulgaris* from the Portuguese coast

Raimundo, J.; Vale, C. (2008). Partitioning of Fe, Cu, Zn, Cd and Pb concentrations among eleven tissues of *Octopus vulgaris* from the Portuguese coast. Ciencias Marinas 34(3), 297-305.

Abstract

Iron, Cu, Zn, Cd and Pb levels were determined in digestive gland, posterior salivary glands, kidneys, gills, gonads, branchial hearts, ink sac, stomach, skin, mantle and arm of thirteen common octopuses, *Octopus vulgaris*, collected in November 2002 at Matosinhos, NW coast of Portugal. No significant ($p>0.05$) differences were found between metal concentrations and size/weight, sex and maturity stage. Medians in digestive gland were one order of magnitude above those in all remaining analysed parts (Cd and Pb), and in all tissues except branchial heart (Fe), posterior salivary glands, gills, mantle and arm (Zn), and arm (Cu). Iron was significantly higher in digestive gland and branchial heart; Cu lower in gonads, mantle and arm; Zn higher in digestive gland and lower in mantle and arm; Cd higher in digestive gland, ink sac and kidney; and Pb higher in digestive gland. Strong Pb-Fe, Cd-Fe and Cu-Zn correlations ($r>0.700$) were obtained in digestive gland, salivary glands, ink sac and stomach. The different metal concentrations in the eleven tissues/organs of octopus are apparently a consequence of the role of metals in metabolic functions (e.g. gonads, ink sac, kidney, gills and salivary glands), although non-essential elements (Cd and Pb) in digestive gland, branchial hearths, kidney and ink sac may be linked to specific ligands or excretory/detoxification mechanisms.

Introduction

The common octopus, *Octopus vulgaris* is a sedentary cephalopod inhabiting coastal waters and thus susceptible to be exposed to contamination (Mangold, 1983). Metal accumulation in its tissues are influenced by local environmental conditions such as, levels in water and food chain, exposure period and temperature, as well as size, sex, and maturity stage (Rossi et al., 1993; Canli and Atli, 2003). Various studies have proved the ability of these specimens to accumulate high levels of essential and non-essential elements, especially in the digestive gland (e.g. Martin and Flegal, 1975; Miramand and Guary, 1980; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante et al., 1998a, b; Raimundo et al., 2004, 2005; Napoleão et al., 2005). Accumulation in other tissues has been related to the presence of molecules vitals to their specific functions, including excretion and detoxifying mechanisms (Blaschko and Himms, 1954; Ghireti-Magaldi et al., 1958; Schipp and Hevert, 1978; Rainbow and Phillips, 1993; Gerpe et al., 2000; Villanueva and Bustamante, 2006).

Despite the large amounts of metals retained in digestive gland and their potential distribution among other tissues and organs, only a few studies have examined the metal partitioning among three to five organs/tissues of octopus (e.g. Miramand and Guary, 1980; Miramand and Bentley, 1992; Nessim and Riad, 2003; Napoleão et al., 2005). This study reports the concentrations of Fe, Cu, Zn, Cd and Pb in eleven tissues of thirteen specimens of *O. vulgaris* captured in November 2002 in the NW coast of Portugal (landed at Matosinhos), which was reported to have elevated levels of metals (e.g., Cd) in water and octopus (Caetano and Vale, 2003; Raimundo et al., 2004).

Material and Methods

Samples

Thirteen common octopuses, *O. vulgaris*, were collected in November 2002 from catches of fishermen in Matosinhos in the NW coast of Portugal (Figure 2.1.1). Specimens were stored in individual plastic bags and immediately frozen onboard in order to minimize mobilization of metals among organs/tissues (Martin and Flegal, 1975). Weight, mantle length and sex were determined for each individual. The sexual maturity was also determined based on the procedures proposed by Guerra (1975). The individuals varied in size and weight over broad ranges (135-210 mm, 844-2609 g, respectively), including males (n=7) and females (n=6) most of them in maturation (stage II). Good relationships between size and weight were found (r=0.89, p=0.001). In the laboratory, digestive gland, posterior salivary glands, kidneys, gills, gonads, branchial hearts, ink sac, stomach, skin, mantle and arm were totally removed under partially defrost conditions without rupture of tissues. Stomach contents were totally removed when present. After separation, individual tissue samples were freeze-dried, grounded and homogenised for the analysis of Fe, Cu, Zn, Cd and Pb.

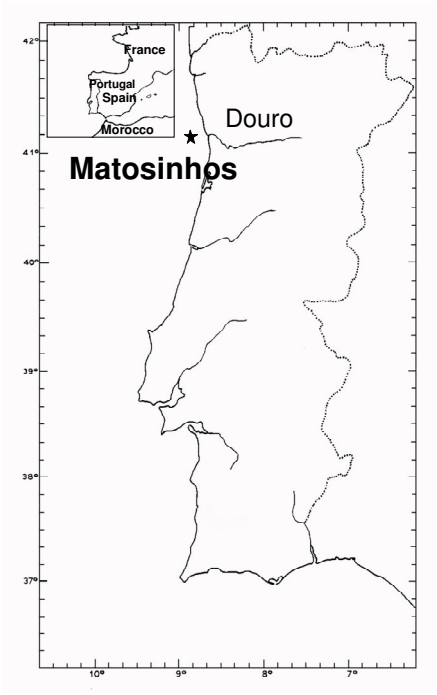

Figure 2.1.1 – Location of the sampling area of *O. vulgaris* in the Portuguese coast: Matosinhos.

Analytical procedure

Approximately 200 mg of dry tissue was digested with a mixture of HNO_3 (sp, 65% v/v) and H_2O_2 (sp, 30% v/v) at 60 ºC for 12 hours, 100 ºC for 1 hour and 1 hour at 80ºC according to the method described in Ferreira et al. (1990). All lab ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Concentrations of Fe, Cu, Zn, Cd and Pb were determined by flame

atomic absorption spectrometry (Perkin Elmer AAnalyst 100) or graphite furnace atomic absorption spectrometry (Perkin Elmer, Zeeman 4110ZL). The accuracy of these analytical methods was assessed by the analysis of international certificate standards, DORM-1, DORM-2 (dogfish muscle), DOLT-1, DOLT-2 (dogfish liver), TORT-1 and TORT-2 (lobster hepatopancreas). Obtained and certified values did not differed significantly ($p>0.05$). Metal concentrations are given as ranges and medians (μg g^{-1}, dry weight).

Statistical analysis

Prior to statistical analysis, metal concentrations and biological parameters were tested for normality and equality of variances. The Mann-Whitney U and Kruskal-Wallis tests were applied to all data in order to detect differences between metal concentrations and biological parameters and tissues. The significance used for statistical analyses was $p<0.05$. The statistical analyses were performed using the SATISTICA 6.0 Statistical Software System.

Results

Metal concentrations

Figure 2.1.2 presents the median, 25 and 75% percentile, minimum and maximum, and the extreme values and outliers, of metal concentrations in the analysed tissues of the octopi (digestive gland, branchial hearts, kidneys, posterior salivary glands, ink sac, gills, stomach, mantle, arm, skin and gonads). Iron concentrations varied from 8.5 μg g^{-1} (arm) to 384 μg g^{-1} (digestive gland); Cu from 8.2 μg g^{-1} (arm) to 762 μg g^{-1} (digestive gland); Zn between 43 μg g^{-1} in the arm and 667 μg g^{-1} found in the digestive gland; Cd presented lower levels in the posterior salivary glands, 0.024 μg g^{-1}, and elevated values in the digestive gland, 185 μg g^{-1}; Pb varied between 0.087 μg g^{-1} (stomach) to 3.5 μg g^{-1} (digestive gland).

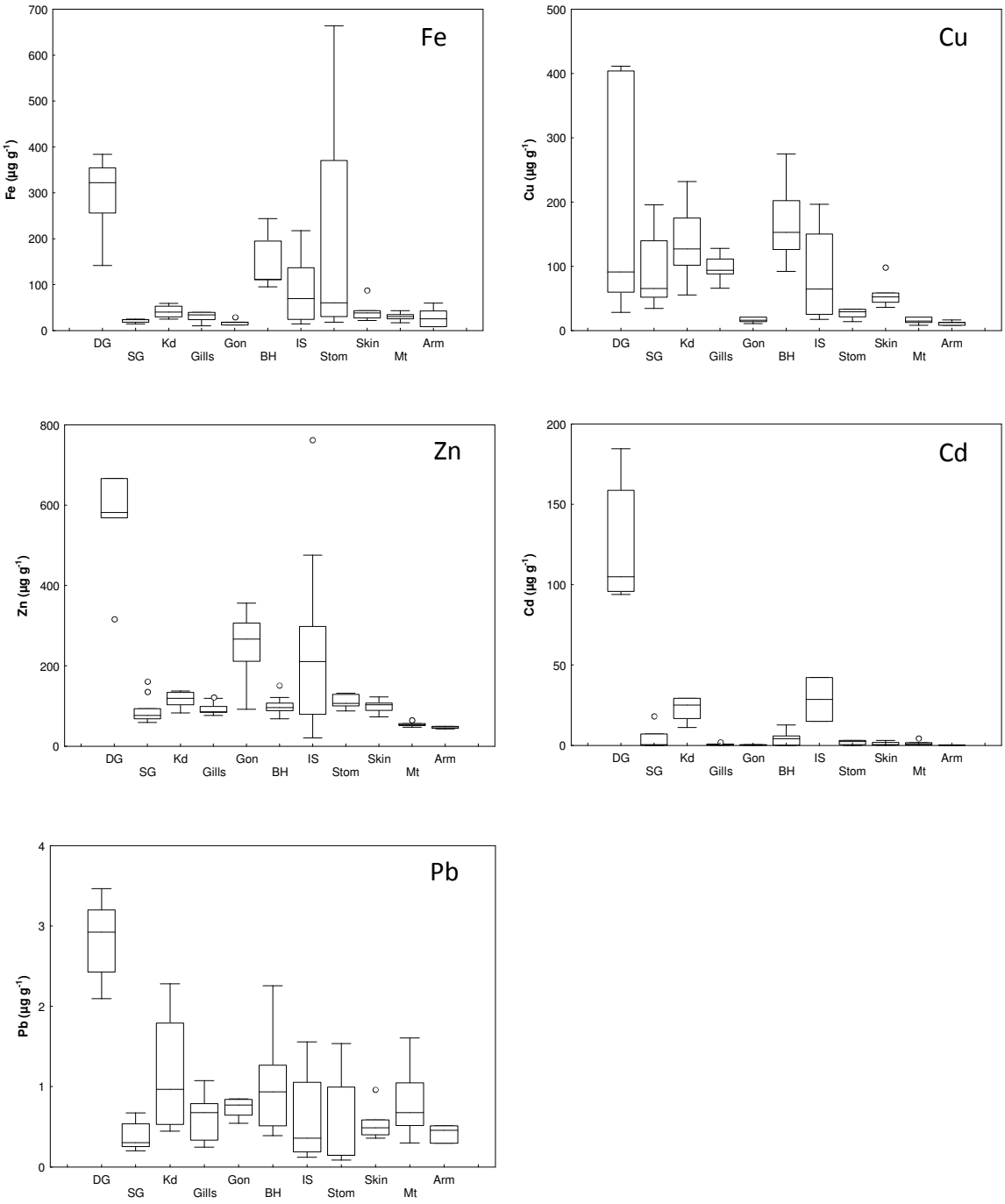

Figure 2.1.2 – Median, 25 and 75% percentile, minimum and maximum, and the extreme values (□) and outliers (•), of Fe, Cu, Zn, Cd and Pb concentrations (µg g^{-1}, dry weight) in the digestive gland (DG), posterior salivary glands (SG), kidneys (Kd), gills, gonads (Gon), branchial hearts (BH), ink sac (IS), stomach (Stom), skin, mantle (Mt) and arm of common octopus, *O. vulgaris*.

Effect of size/weight and sex on metal concentration

Concentrations of Fe, Cu, Zn, Cd and Pb in the analysed tissues showed no significant ($p>0.05$) differences with the size/weight, suggesting that growth has minor effects on metal accumulation within the ranges of size/weight of the sampled individuals.

Metal–metal correlations

The correlations between metal concentrations were only obtained in digestive gland, posterior salivary glands, ink sac and stomach (Table 2.1.1). The Pb-Fe relationships were found in all these tissues/organs except in salivary glands. Cadmium was correlated to Fe, Cu and Zn in salivary glands.

Table 2.1.1 – Metal-metal correlations (r) and associated probabilities (a – 0.05; b – 0.01; c – 0.001) in the digestive gland, salivary glands, ink sac and stomach of the *O. vulgaris* captured in Matosinhos.

		Fe	Pb	Cd	Cu	Zn
Digestive gland	Pb	0.851 [b]	-	0.939 [c]	-	-
Ink sac	Pb	0.918 [c]	-	-	-	-
Stomach	Pb	0.729 [a]	-	-	-	-
Salivary glands	Cd	0.922 [c]	0.700 [a]	-	0.968 [c]	0.726 [a]

Differences of Fe, Cu, Zn, Cd and Pb among tissues

Levels of Fe in the digestive gland and branchial heart were significantly higher than in all the analysed tissues, except ink sac. Stomach, kidney, skin, gills, mantle and arm exhibited similar values (Figure 2.1.2). Copper levels in gonads, mantle and arm were significantly lower than in the other tissues, with the exception of stomach. Digestive gland exhibited a high variability raging from 28 to 762 μg g^{-1}. Zinc in the digestive gland was statistically higher than in mantle and arm (low) and in all the remained tissues (intermediate values). Gonads exhibited significantly different values from the other tissues, except ink sac. Posterior salivary glands, kidneys, gills, branchial hearts, stomach and skin presented similar concentrations. The partitioning of Cd was clearer since levels in the digestive gland, ink sac and kidney were significantly higher than in all the other analysed tissues. Digestive gland presented also significantly higher levels of Pb. Salivary glands showed lower Pb than the other tissues, with the exception of skin, ink sac and stomach.

Discussion

Comparison with other works

The lack of relationships between size/weight and sex on metal concentrations are in line with other works reporting similar concentrations in small and large individuals of other octopus species, *Graneledone sp.* and *Benthoctopus thielei* (Bustamante et al., 1998a) and of the same species (Seixas et

al., 2005). However, *Octopus salutii* showed a negative relationship between Cd concentrations and weight (Storelli and Marcotrigiano, 1999). Furthermore, metal concentration did not differed between males and females, which agrees with other works on cephalopods (Miramand and Bentley, 1992; Bustamante et al., 1998a; Barghigiani et al., 2000) though higher levels of Fe have been detected in females (Seixas et al., 2005). Due to the absence of relationships, the eleven specimens were treated independently of their size and sex. Comparing to other works, the Fe, Cu, Zn and Pb levels fall within the interval concentrations of each tissue reported for *O. vulgaris* from various coastal waters (Table 2.1.2). The Cd levels in tissues of octopus from the NW Portuguese coast exceeded some of the values reported in the literature, which may be attributed to the high availability of Cd in coastal waters associated with river inputs (Caetano and Vale, 2003; Raimundo et al., 2004).

Table 2.1.2 – Comparison of Fe, Zn, Cu, Cd and Pb levels (µg g^{-1}, dry weight) in the digestive gland, branchial hearts, gills, gonads, posterior salivary glands, mantle, arm and mantle skin of *O. vulgaris* from Matosinhos (present study) with data from the literature.

Organ/tissue of *O. vulgaris*	Fe	Cu	Zn	Cd	Pb	Authors
			µg g^{-1}			
Digestive Gland						
	142-384	28-762	81-667	94-185	0.71-3.5	Present study
	-	139-3140	200-14721	19-761	0.037-44	Raimundo et al. (2004)
	-	137-1465	198-14721	20-269	-	Raimundo et al. (2005)
	790±343	1768±1010	1463±726	-	6.9±3.2	Napoleão et al. (2005)
	700±130	2500±700	1450±400	50±10	-	Miramand and Guary (1980)
	130-875	8.6-286	113-437	34-218	-	Soldevilla (1987)
Branchial hearths						
	95-1270	92-274	68-386	0.032-88	0.39-3.9	Present study
	650±150	500±40	65±15	0.08±0.04	-	Miramand and Guary (1980)
	577±323	188±68	81±23	-	8.1±5.0	Napoleão et al. (2005)
	41-69	14-58	58-121	2.5-3.8	8.3-9.4	Nessim and Riad (2003)
Gills						
	10-252	66-128	76-122	0.16-22	0.25-1.1	Present study
	40±28	113±47	72±17	-	-	Napoleão et al. (2005)
	19-87	92-253	44-94	17	-	Soldevilla (1987)
	11-16	11-21	24-38	1.2-2.0	2.1-4.4	Nessim and Riad (2003)
Gonads						
	12-29	11-46	92-357	0.067-2.0	0.54-0.85	Present study
	30±10	50±8	360±200	0.1±0.04	-	Miramand and Guary (1980)
	18-28	27-42	8.1-26	1.0-2.6	1.5-15	Nessim and Riad (2003)
Posterior salivary glands						
	15-79	34-195	59-182	0.024-18	0.20-0.67	Present study
	19-32	12-36	56-116	3.1-4.4	2.0-23	Nessim and Riad (2003)
Mantle						
	17-43	8.3-62	47-64	0.25-4.4	0.30-1.6	Present study
	11-84	17-106	41-186	23	-	Soldevilla (1987)
	30±5	26±1	70±30	0.08±0.04	-	Miramand and Guary (1980)
	8.7-48	13-99	59-193	0.13-11.1	0.056-4.3	Raimundo et al. (2004)
	24±15	30±19	76±22	-	-	Napoleão et al. (2005)
	14-81	12-68	67-121	0.27-3.3	-	Raimundo et al. (2005)
	11-16	13-20	5.2-13	1.3-1.8	1.8-7.4	Nessim and Riad (2003)
Arm						
	8.5-60	8.2-16	43-66	0.11-0.26	0.29-2.2	Present study
	14-58	5.5-72	53-107	0.035-1.0	-	Raimundo et al. (2005)
	40-50	4-203	50-300	20	3-4	Seixas et al. (2005)
	5.7-49	5.5-20	64-119	0.053-1.3	0.058-0.87	Raimundo et al. (2004)
	10-76	7.8-36	53-75	1.8	-	Soldevilla (1987)
Skin						
	22-87	36-98	73-123	0.16-3.2	0.36-1.0	Present study
	30±10	50±4	50±10	0.04±0.01	-	Miramand and Guary (1980)
	11-19	12-18	11-16	1.4-2.3	3.6-7.4	Nessim and Riad (2003)

Accumulated metals in organs/tissues

***Digestive gland*.** It exhibited the highest metal concentrations. Medians were one order of magnitude above those in all remaining analysed parts (Cd and Pb), and in all tissues except branchial heart (Fe), posterior salivary glands, gills, mantle and arm (Zn), and arm (Cu). The elevated levels corroborate the presence of efficient mechanisms to store metals in this organ (e.g. Martin and Flegal, 1975; Miramand and Guary, 1980; Smith et al., 1984; Miramand and Bentley, 1992; Bustamante et al., 1998a, b; Bustamante et al., 2002; Raimundo et al., 2004; Napoleão et al., 2005; Seixas et al., 2005). In cephalopods, the ratio between metal concentrations in digestive gland and muscle has been used to separate elements in three groups (Miramand and Bentley, 1992): poorly concentrated (ratio <10); moderately concentrated (10<ratio<50); and highly concentrated (ratio>50). The calculation of this ratio showed that Pb (ratio: 4-6) was poorly, Fe (ratio: 11-13), Cu (ratio: 17-20) and Zn (ratio: 11-12) moderately and Cd (ratio: 98-585) highly concentrated. The strong association of Cd with lysosomes and cytosolic proteins (Finger and Smith, 1987; Castillo and Maita, 1991; Bustamante et al., 2002) appears to be emphasised in octopus from the NW region of Portugal probably due to higher availability (Caetano and Vale, 2003).

Gills and stomach. The high levels of Cu in gills are in line with other studies (Soldevilla, 1987) that linked its abundance with hemocyanin, in which Cu is one of the main components of this respiratory pigment (Soldevilla, 1987; Villanueva and Bustamante, 2006; Craig and Overnell, 2003). Iron, Zn and Cd were higher in the stomach, while Cu and Pb showed increased concentrations in gills, suggesting preferential uptake through food or water.

Mantle, arms and skin. As found in previous work (Raimundo et al., 2004), levels of Cd and Pb in mantle were significantly higher than in arm, pointing to efficient binding sites in that tissue. Different protein composition (Kariya et al., 1986) may contribute to the distinct accumulation. Iron, Zn and Cu were more concentrated in skin than mantle and arms. These differences were found in other studies (Miramand and Guary, 1980; Miramand and Bentley, 1992), suggesting changes on environmental availability. Since these tissues are consumed by humans, levels were compared to the safety limit established by European Commission (1.0 μg g^{-1}, ww of Cd and Pb, Journal of EU Communities 2001, EC rule no. 466/2001). Tissues of only four of the thirteen analysed specimens presented Cd and Pb levels above the limits (Cd=1.1, 1.2 and 3.1 μg g^{-1}; and Pb=1.5 μg g^{-1}).

Salivary gland. Copper was the main concentrated metal in this gland containing a mixture of several toxic substances used to kill the prey (Kanda et al., 2003). The abundant Cu in *O. vulgaris* has been observed by Nessim and Riad (2003) and may be related to Cu-amine oxidase (Blaschko and Himms, 1954).

***Branchial hearts*.** The high levels of Fe and Cu agree with the findings of Miramand and Guary (1980) for the same species in the coast of Monaco. The presence of adenochromes may be responsible for Fe complexation (Ghireti-Magaldi et al., 1958), and of respiratory pigment hemocyanin by the elevated Cu (Miramand and Guary, 1980). Branchial hearts presented also enhanced levels of Cd and Pb that may be

related to storage and detoxification mechanisms (Guary and Fowler, 1982) linked to the circulatory and excretory functions of these organs (Schipp and Hevert, 1978; Villanueva and Bustamante, 2006).

***Kidney*.** It concentrated elevated levels of Pb, as well as Cd and Cu which have been registered in *Eledone cirrhosa* and *Sepia officinalis* (Miramand and Bentley, 1992) and *O. vulgaris* (Miramand and Guary, 1980) and interpreted as the result of its excretory function (Rainbow and Phillips, 1993).

Ink sac. The high levels of Cu and Zn may be associated with melanin (Bustamante et al., 1998a), and Cd related to excretory pathway of the ink.

Gonads. It presented enhanced levels of Zn, being in accordance to different studies (Miramand and Guary, 1980; Miramand and Bentley, 1992; Bustamante et al., 1998a; Gerpe et al., 2000) and related to high quantities of Zn-containing enzymes and metalloproteins (Gerpe et al., 2000).

In short, metal concentrations differed considerably among the eleven tissues/organs of octopus, apparently as a consequence of the role of metals in metabolic functions (e.g. gonads, ink sac, kidney, gills and salivary glands). The presence of non-essential elements (Cd and Pb) in digestive gland, branchial hearths, kidney and ink sac may be linked to specific ligands or excretory/detoxification mechanisms.

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Chapter 2.2

Total lead and its stable isotopes in digestive gland of *Octopus vulgaris* as a fingerprint

Raimundo, J., Vale, C., Caetano, M., Cesário, R., Moura, I. (2009). Total lead and its stable isotopes in digestive gland of *Octopus vulgaris* as a fingerprint. Aquatic Biology 6(1), 25-30.

Abstract

Forty seven *Octopus vulgaris* were captured between November 2005 and September 2006 in two areas of the Portuguese coast, near Matosinhos - A (NW coast) and Olhão - B (S coast), and digestive glands were analysed for total lead and its stable isotopes. The same determinations were performed in 22 samples of surface sediments from the two areas. Lead concentrations in the digestive gland of specimens from area B (2.8 - 13.0 μg g^{-1}) exceeded the values found in area A (1.3 - 8.3 μg g^{-1}). A similar pattern was found for the Pb isotopic ratios: $^{206}Pb/^{207}Pb$ = 1.173 - 1.185 (A), 1.165 - 1.172 (B); $^{206}Pb/^{208}Pb$ = 0.476 - 0.487 (A) and 0.318 - 0.483 (B). The different signatures of the digestive glands are in line with those observed in the surface sediments of the two coastal areas (e.g. $^{206}Pb/^{207}Pb$ = 1.179 - 1.207 (A), 1.171 - 1.181 (B)). However, lead isotopic signature of octopus was less radiogenic than sediments. Because the octopus has a short life span (up to 24 months) the signature reflects recent sources of Pb which have less radiogenic signature. Lead signature of surface sediments tends to integrate the record of the last years or decades, due to the frequent resuspension of upper layer of coastal sediments. The mixing of sediments deposited along those periods of time results in the increasing of Pb isotopic ratios (more radiogenic). The consistent differences between the two areas, either in sediments and octopus, points that Pb isotopic signature may provide a useful tool to distinguish octopus populations.

Introduction

The marine biogeochemical cycle of lead (Pb) has been greatly affected by human activities in the last century (Komárek et al., 2008). Industrial emissions and gasoline exhaust led to an increase of Pb deposition into the marine environment (Alleman et al., 2000). In the past, understanding of Pb bioaccumulation relied mainly on concentration measurements. Because Pb isotope ratios vary according to the origin of this element, the inclusion of these values in environmental studies allowed distinguishing the pathway of Pb from distinct sources (Komárek et al., 2008). There are four stable isotopes with mass numbers: ^{204}Pb (primordial), ^{206}Pb, ^{207}Pb and ^{208}Pb (radiogenic). The last three isotopes are products of the radioactive decay of ^{238}U, ^{235}U and ^{232}Th, respectively (Scheuhammer and Templeton, 1998). The isotopic composition of anthropogenic and natural Pb generally differs and it is seldom affected by kinetic processes (Gobeil et al., 2001).

The high persistence of Pb in abiotic compartments and its accumulation in living organisms has stimulated the determination of baseline values and spatial distribution of Pb concentration in sediments and biota, as well as the study of its behaviour in the coastal zone (e.g. Prego and Cabelo-Garcia, 2004). In the last decade, determination of Pb concentration in sediments has been coupled with of Pb isotope ratios to better understand the fate of this element within the coastal ecosystems. However, the Pb isotopic signature in marine organisms has been poorly documented. Only few works have pointed out that biological samples can provide a fingerprint for sources of Pb (Spencer et al., 2000; Ip et al., 2005).

Octopus vulgaris, common octopus, is a benthonic species, exclusively neritic, with the exception of the larval phase that is planktonic. They have a short live span, fast growth rates and high reproductive

potential (Rocha et al., 2001). This species is normally distributed on rocky, sandy and muddy bottoms (Mangold, 1983). Octopus has been long considered of cosmopolitan occurrence in temperate and tropical seas (Roper et al., 1984), although a possible occurrence of cryptic species among *O. vulgaris*-like octopods is also reported (Guerra et al., 1999). Thus, the distribution of *O. vulgaris* in a strict sense may be restricted to the Mediterranean Sea and eastern Atlantic Ocean (Mangold, 1983). Octopus undergo in vertical seasonal migrations, being close to the shore for reproduction (Mangold and Boletzeky, 1973).

Bioaccumulation studies have reported that storage of Pb in cephalopods occurs mainly in the digestive gland (e.g. Miramand and Bentley, 1992; Nessim and Riad, 2003; Seixas et al., 2005; Bustamante et al., 2008; Raimundo and Vale, 2008). Furthermore, accumulated Pb levels appear to respond to its availability in water and food (Bustamante et al., 1998; Raimundo et al., 2004, 2005; Napoleão et al., 2005). However, to our knowledge no attempt was done to clarify whether Pb accumulated values in digestive gland reflect the anthropogenic versus natural origin of Pb.

Contrasting geomorphologic features and oceanographic conditions were reported for the Portuguese coast (Fiuza, 1983): the typology of the NW region is an exposed coast characterized by several estuarine systems crossing the shore, while the South region has been classified as sheltered coast with extensive inner coastal lagoons. The Iberian peninsula is crossed by a giant massive sulphide deposit in the southern region (Iberian Pyrite Belt), mined since the Roman Age (Palanques et al., 1995), which has a relatively homogeneous Pb isotopic signature (Marcoux, 1998). Water surveys pointed to contrast availability of trace elements between the NW and Southern coastal waters (Caetano and Vale, 2003). River flow regime and pyrite belt location were invoked as major factors influencing those differences. A similar geographic contrast was found for Zn, Pb, Cd and Hg concentrations in digestive gland of *Octopus vulgaris* (Raimundo et al., 2004; Napoleão et al., 2005; Seixas et al., 2005): enhanced levels of Pb, Hg, and Zn in individuals from the south coast and higher accumulation of Cd in specimens captured in NW stations.

The two areas are, therefore, privileged to test the hypothesis if Pb isotopic signature in digestive gland of octopus reflects the Pb sources, and whether ratios are useful to characterise octopus populations. This study contains data on total and stable Pb isotopes in digestive gland of *O. vulgaris* from NW and South areas of Portugal, as well as Al and Pb concentrations and stable Pb isotopes in sediments used to distinguish Pb signatures between the two studied areas.

Material and Methods

Samples. Forty seven common octopuses, *Octopus vulgaris*, were collected between November 2005 and September 2006 from commercial catches landed in Matosinhos (NW coast) and Olhão (South coast). Specimens were captured within two areas of 6 miles radius centred at Matosinhos (area A) and Olhão (area B) (Figure 1). Total body weight, mantle length and sex were determined in each individual. Specimens were stored in individual plastic bags and frozen (-80 ºC) in order to minimize mobilization of metals among organs/tissues (Martin & Flegal 1975). In the laboratory, digestive gland was totally

removed under partially defrost conditions without rupture of the tissue, freeze-dried, grounded and homogenised.

Surface sediments were collected in February 2006 in the areas A (12 samples) and B (10 samples), using a Van-Veen grab onboard of the research vessel Noruega. The top 5-cm sediment layer was sampled. Each sediment sample was oven-dried to constant weight at 40 ºC, sieved through a 2-mm mesh and grounded with an agate mortar.

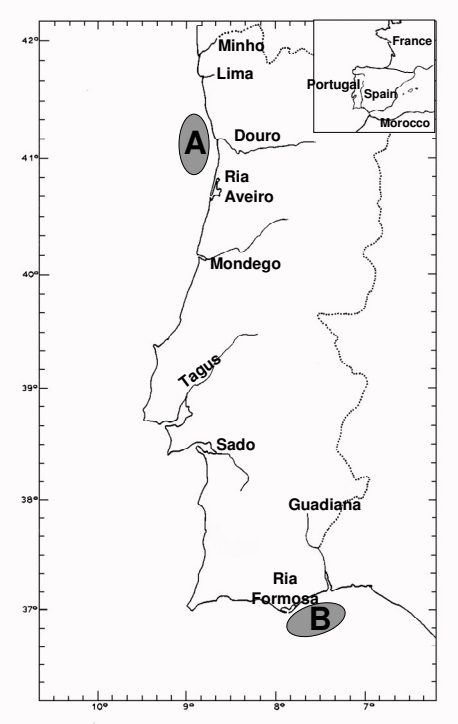

Figure 2.2.1 – *Octopus vulgaris*. Location of the two sampling sites in the Portuguese coast: A (Matosinhos) and B (Olhão).

Analytical methodology

Sample pre-treatment. Samples of digestive gland (≈200 mg) were digested with a mixture of HNO_3 (sp, 65 % v/v) and H_2O_2 (sp, 30 % v/v) at 60 ºC for 12 hours, 100 ºC for 1 hour and 1 hour at 80 ºC according to the method described in Ferreira et al. (1990). Two mineralization procedures were used for sediment samples: 1) digestion for Al quantification using HF (sp, 40 % v/v), Aqua Regia (HCl-36 %:HNO_3-65 %; 3:1) and H_3BO_4 following the method described by Rantala & Loring (1975); and 2) mineralization for analysis of Pb concentration and stable Pb isotopes by using the first step of the previous method, evaporated to near dryness and elute with HNO_3 (double-distilled) and Milli-Q water (18.2 MΩ.cm) (Caetano et al. 2007). Procedural blanks were prepared using the same analytical procedure and reagents, and included within each batch of 10 samples.

Methods. Aluminium was analysed by flame atomic absorption spectrometry (Perkin Elmer AA100) with a nitrous oxide-acetylene flame and concentrations determined with the standard addition method. Total Pb concentration and stable Pb isotopes (^{206}Pb, ^{207}Pb and ^{208}Pb) were determined in the same samples but in separate runs using a quadropole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. A 7-points calibration within a range of 1 to 100 µg L^{-1} was used to quantify total Pb concentration. The precision and accuracy of the Pb concentration measurements, determined through repeated analysis of references materials (BCSS1 and MESS3 for sediment and TORT1 and TORT2-lobster hepatopancreas for organisms), using ^{115}In as internal standard, was better than 2 % (Table 2.2.1). Procedural blanks always accounted for less than 1 % of the total lead in the samples. For Pb isotope determinations, between every two samples, corrections for mass fractionation were applied using NIST-SRM981 reference material. The Pb isotopic composition of procedural blanks did not influence significantly the ^{206}Pb/^{207}Pb and ^{206}Pb/^{208}Pb ratios measured in all samples. The coefficients of variation of the NIST-SRM981 reference material obtained in between-batch external quality control were 0.37 % for ^{206}Pb/^{207}Pb and 0.22 % for ^{206}Pb/^{208}Pb ratios.

Table 2.2.1 – Lead (µg g^{-1}, dry weight) and Al (%, dry weight) concentrations of lobster hepatopancreas (TORT-1 and TORT-2) and marine sediments (BCSS-1 and MESS-3) (NRCC) obtained in the present study and certified values.

Standard	Pb	Al
	(µg g^{-1})	(%)
TORT-1		
Obtained	9.4±1.9	-
Certified	10.4±2	-
TORT-2		
Obtained	0.43±0.27	-
Certified	0.35±0.13	-
BCSS-1		
Obtained	23.0±3.7	6.56±0.17
Certified	22.7±3.4	6.26±0.22
MESS-3		
Obtained	22.1±3.1	9.17±0.23
Certified	21.9±1.2	8.59±0.23

Statistical analysis

Prior to statistical analysis, metal concentrations and biological parameters were tested for normality and equality of variances. The non-parametric test, Kruskal-Wallis test (KW-H), was applied to all data in order to detect differences between metal concentrations and biological parameters and in the two studied areas. The statistical analyses were performed using the STATISTICA 6.0 Statistical Software System.

Results

Biologic parameters in octopus

The octopus sampled in area A included 13 males and 11 females, and the specimens in area B 12 males and 11 females. Size and weight of the sampled individuals varied over broad ranges: area A, 125 - 170 mm and 578 - 1433 g; area B, 113 - 165 mm and 698 - 1520 g. Size, weight and sex were not significantly ($p > 0.05$) different in the specimens from the two areas. Differences between sampling periods have also no statistical validity ($p > 0.05$).

Lead concentrations and isotopic ratios in digestive gland

Levels of total Pb differed significantly between the two areas (Figure 2.2.2): 1.3 - 8.3 µg g^{-1} in individuals from A, and 2.8 - 13.0 µg g^{-1} from B. Lead isotopes ratios in specimens from area A ($^{206}Pb/^{207}Pb$ = 1.173 - 1.185; $^{206}Pb/^{208}Pb$ = 0.476 - 0.487) were significantly ($p < 0.001$) higher than from area B ($^{206}Pb/^{207}Pb$ = 1.165 - 1.172 and $^{206}Pb/^{208}Pb$ = 0.318 - 0.483). No statistical differences ($p > 0.05$) were found between Pb concentration or the lead isotopic ratios and the measured biological parameters. Differences between sampling periods have also no statistical validity.

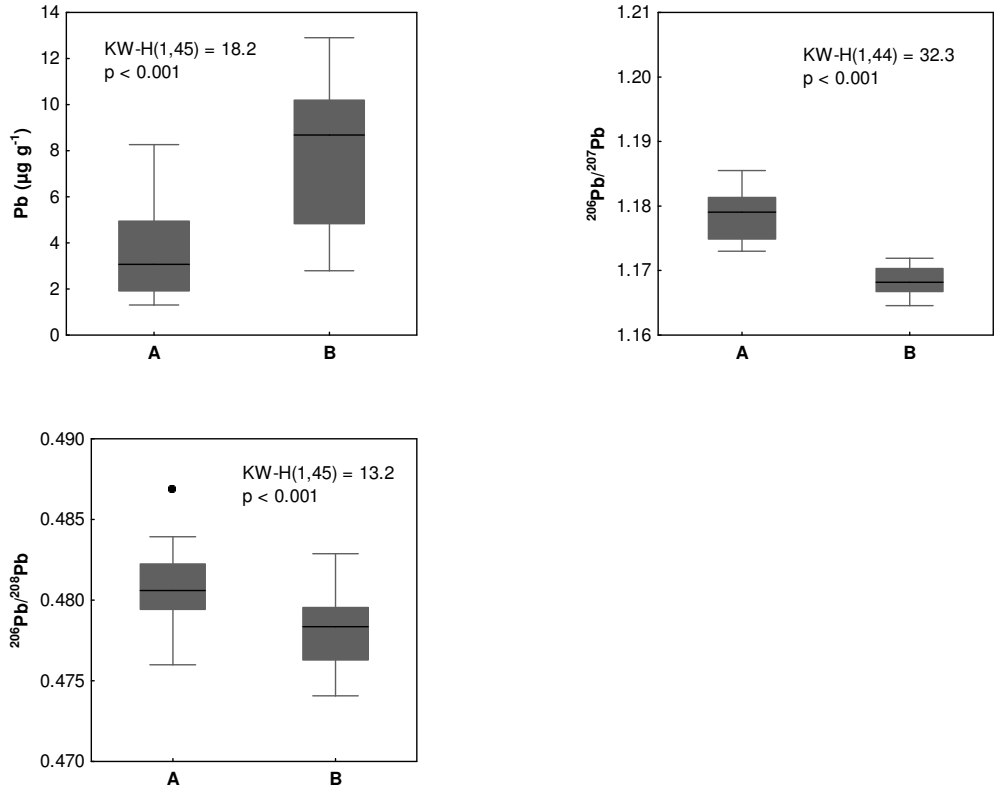

Figure 2.2.2 – *Octopus vulgaris.* Median, 25% and 75% percentiles, minimum and maximum, outliers (•), Kruskal-Wallis test (KW-H) and p-values of Pb concentrations (µg g^{-1}, dry weight) and $^{206}Pb/^{207}Pb$ and $^{206}Pb/^{208}Pb$ ratios in the digestive gland of common octopus collected in two areas of the Portuguese coast (A and B).

Aluminium and lead concentrations in sediments

Aluminium content in surface sediments from areas A and B ranged within broad intervals: 1.9 - 6.8% and 0.58 - 8.7%, respectively. These results are indicative that sediments sampled in the two areas presented a wide mixture of coarse (low Al content) and fine-grained particles (high Al content). The sediment samples of the two areas showed no significant ($p>0.05$) differences on the Al content. When metal concentrations are compared in one sediment set containing different grain size it is recommended to normalise levels to Al in order to minimize differences associated with sediment nature (Windom et al. 1989). For this reason Pb concentrations in this study were normalized to Al content. Lead concentrations and Pb/Al ratios in surface sediments are presented in figure 3, as median, percentile 25% and 75%, maximum and minimum values. Lead concentrations varied in a wide range, 8.1 - 25 µg g^{-1} (A) and 7.9 - 57 µg g^{-1} (B), being levels in area B significantly ($p<0.05$) higher than in area A. Normalizing Pb to Al separated pronouncedly the two areas, meaning that elevated Pb concentrations in area B are not due to a more abundant fine fraction. The values of Pb/Al in area B (3.5 - 15.0x10^{-4}) were significantly ($p<0.001$) higher than those from area A (3.3 - 5.1x10^{-4}).

Lead isotopic ratios in sediments

Surface sediments from the area A showed a more radiogenic signature of $^{206}Pb/^{207}Pb$ (1.179 - 1.207) than those from area B (1.171 - 1.181). Moreover, a broader range of this ratio was found in sediments from area A (Figure 2.2.3) at a significant level ($p<0.001$). However, no significant differences of $^{206}Pb/^{208}Pb$ ratios were found between the two areas ($p>0.05$).

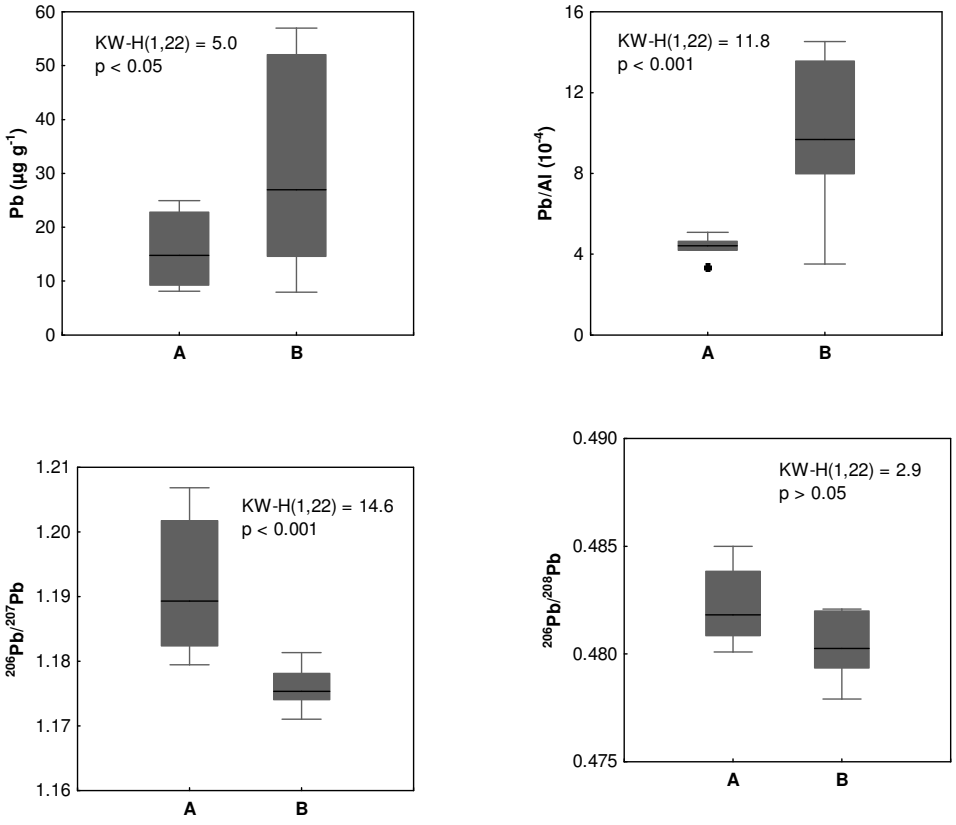

Figure 2.2.3 – Median, 25% and 75% percentiles, minimum and maximum, outliers (•), Kruskal-Wallis test (KW-H) and p-values of Pb concentrations (µg g^{-1}, dry weight), Pb/Al (10^{-4}) and $^{206}Pb/^{207}Pb$ and $^{206}Pb/^{208}Pb$ ratios in the surface sediments collected in two areas of the Portuguese coast (A and B).

Discussion

The broad range of Al content in surface sediments indicates the existence of a wide combination of coarse and fine-grained materials in the two study areas. Despite of that variability, Pb/Al ratios were consistently higher in the area B. The elevated ratios appear to result mainly from the geologic feature - Iberian Pyrite Belt - since anthropogenic Pb sources are minor. Indeed, the narrow range of Pb isotopic signature of sediments ($^{206}Pb/^{207}Pb$ = 1.171 - 1.181) matches with those found by Caetano et al. (2007) in sediments from Guadiana River, the main river that crosses the sulphide deposit area ($^{206}Pb/^{207}Pb$ = 1.172 ± 0.003). The obtained Pb isotopic signature in coastal sediments from the area B points to a mixing of particles derived from the pyrite region and pre-industrial sediments with minor inputs of anthropogenic

Pb (Caetano et al., 2007). In contrast, sediments from area A exhibited a broader range of $^{206}Pb/^{207}Pb$ ratios suggesting that Pb concentration in sediments was influenced by Pb from various origins. The observed $^{206}Pb/^{207}Pb$ ratios (1.179 - 1.207) were comprised between values reported for sediments contaminated by industrial effluents (1.166 - 1.170; Sundby et al., 2005) and pre-pollution Pb signature as recorded in the NW Spain (1.235; Kylander et al., 2005) or in pre-industrial sediments from North Atlantic (1.197 - 1.220; Sun, 1980). Area A receives the discharges of the Douro River, after crossing an extensive rural area, and of the urban effluents of Porto (Caetano and Vale, 2003). The Pb isotopic signature of coarse and fine sediments from the area A may thus mirror the mixture of high radiogenic background Pb and low radiogenic contaminant Pb emissions of alkyllead gasoline (1.06 - 1.09; Gobeil et al., 2001).

Lead concentrations in the digestive gland of *Octopus vulgaris* captured in the two areas ranged within the intervals observed in previous works (Raimundo et al., 2004; Napoleão et al., 2005; Raimundo et al., 2008). Interestingly, the elevated concentration of Pb in sediments (area B), as well as of Pb/Al ratios, matches with the increased values in digestive gland of specimens from the same area. This response to the environmental availability is consistent with findings of other investigations with cephalopods (Bustamante et al., 1998; Koyama et al., 2000; Raimundo et al., 2004; Napoleão et al., 2005). Our results do not allow evaluating the preferential pathway of accumulation however, given the sedentary habits of octopus, both water and food should be considered vehicles to Pb uptake. It is expected that Pb in sediments influences the levels existing in benthic organisms that constitute the octopus diet, including crabs and bivalves (Mangold, 1983). The uptake of Pb from different pathways presupposes the accumulation of Pb with distinct signatures. Thus the observed signature is an integration of all local sources. Specimens from area B exhibited less isotopic ratios ($^{206}Pb/^{207}Pb$ ratios = 1.165 - 1.172) than from area A (1.173 - 1.185), mirroring the Pb signature of each area. This parallelism has been rarely reported for marine organisms.

However, isotopic signature in octopus and sediments did not show the same range of values. These differences are consistent with findings from Ip et al. (2005) that showed lower lead isotopic ratios in molluscs, crustacean and fish than in sediments. Because octopus has a short life span (up to 24 months, Mangold, 1997), lead isotopic ratios in digestive gland should reflect recent sources of Pb that in comparison to the past have less radiogenic signature. The two coastal areas are frequently subjected to suspension of surface sediments and settling events due to wave or wind storms. As a result, the Pb isotopic signature of the collected sediments tends to integrate the record of the last years or decades. Therefore the Pb signature in sediments showed higher isotopic ratios than in octopus tissues. The hypothesis of segregate accumulation of Pb isotopes by *O. vulgaris* or differential isotope detoxification is out of the scope of this work. These results indicate that Pb isotopic signature in digestive gland of octopus by reflecting the Pb sources offers a useful tool to distinguish octopus populations.

Acknowledgments

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Chapter 2.3

Relations between mercury, methyl-mercury and selenium in tissues of *Octopus vulgaris* from the Portuguese Coast

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.

Abstract

Mercury, methyl-mercury (MeHg) and selenium were determined in digestive gland and mantle of *Octopus vulgaris*, from three areas of the Portuguese coast. To our knowledge these are the first data on MeHg in cephalopods. Concentrations were higher in the digestive gland and percentage of MeHg in mantle. Enhanced Hg and MeHg levels were obtained in digestive gland of specimens from Olhão (3.1-7.4 and 2.0-5.0 μg g^{-1}, respectively). Differences between areas may be partially related to Hg availability. Relationships between concentrations in mantle and digestive gland pointed to proportional increases of Hg and MeHg in tissues of specimens from Matosinhos and Cascais, but relatively constant values in mantle of individuals from Olhão (higher contamination). Se:Hg molar ratio in digestive gland was 32 and 30 in octopus from Matosinhos and Cascais, respectively, and 5.4 from Olhão. The proximity to the unit suggests demethylation as response to elevated MeHg levels in digestive gland.

Introduction

Mercury (Hg) is one of the most hazardous environmental pollutants that is known not to play any essential role in biochemical functions (Jackson, 1998). The most toxic form, methyl-mercury (MeHg) is produced in aquatic environment by bacteria and biomagnifies through the food web as result of elimination being slower than uptake (Gilmour et al., 1992; Kidd et al., 1995; Mason and Benoit, 2003). Fish uptake Hg mainly through the diet (Bloom, 1992; Hall et al., 1997; Porcella, 1994) with MeHg being the form predominantly stored in muscle tissue (Harris et al., 2003; Amlund et al., 2007). The incorporation of Hg may vary with biological (growth rate, size, sex), ecological (food, habitat) and environmental factors (Hg availability, methylation rate, primary productivity) (Harmelin-Vivien et al., 2009). The literature reflects disagreement on the effect of some factors, such as size and sex, on accumulation in octopus (Barghigiani et al., 1991; Monteiro et al., 1992; Rossi et al., 1993; Storelli and Marcotrigiano, 1999; Raimundo et al., 2004; Seixas et al., 2005a; Bustamante et al., 2006; Pierce et al., 2008). The complexity and interactions of factors influencing bioaccumulation explain the variety of results obtained for different species and environments (Trudel and Rasmussen, 2006; Magalhães et al., 2007; Schwindt et al., 2008). The antagonistic action of selenium (Se) against the toxicity of mercury forms in aquatic organisms has been proposed in last years (Chen et al., 2001; Belzile et al., 2006). Selenium seems to have a blocking mechanism in methylation by the precipitation of HgSe or to contribute to MeHg demethylation in the liver (Yang et al., 2008).

The common octopus *Octopus vulgaris* is a voracious predator normally distributed on rocky, sandy and muddy bottoms. It is characterized by fast growth rates and a short lifespan (Mangold and Boletzky, 1973; Guerra, 1975; Mangold, 1983; Rocha et al., 2001). The digestive gland of octopus accumulates high levels of Hg and other elements, resulting from the intrinsic capacity of this organ to storage metals, which suggests a major role of this organ in detoxification and assimilation processes (Bustamante et al., 1998a, 2000, 2002; Raimundo et al., 2004; Seixas et al., 2005a, b; Bustamante et al., 2006; Storelli et al., 2006). Selenium levels have been reported in octopus but no relationships with

mercury were searched (Seixas et al., 2005b). Only one work reported the concentrations of organic-Hg in cephalopods (Bustamante et al., 2006).

This paper reports the levels of total Hg, total Se and, to the best of our knowledge, for the first time the concentrations of MeHg in digestive gland and mantle of *O. vulgaris*. Specimens were caught in three areas of the Portuguese coast, Matosinhos, Cascais and Olhão. Data from INAG (National Institute of Water) and Canário et al. (e.g. 2007) have documented different levels of Hg in the surroundings of abovementioned areas.

Material and Methods

Samples

Thirty three common octopuses, *O.vulgaris*, were collected in February and October 2006 from commercial catches landed in Matosinhos (n=11), and Olhão (n=12) and in February 2008 in Cascais (n=10), situated in the NW, W and SE coast of Portugal, respectively (Figure 2.3.1). Total body weight, mantle length (size) and sex were determined in each individual. Specimens were stored in individual plastic bags and frozen (-80 ºC) in order to minimize mobilization of metals among organs/tissues (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. After separation, individual tissue samples were freeze-dried, ground and homogenised for the analysis of total mercury, methyl-mercury and total selenium.

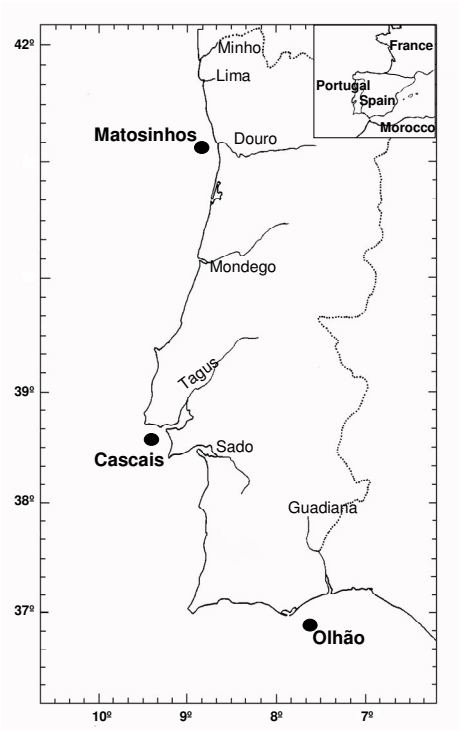

Figure 2.3.1 – Location of the three areas of capture of *Octopus vulgaris* in the Portuguese Coast: Matosinhos, Cascais and Olhão.

Analytical methodology

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).

For Se determinations, samples were previously digested in Teflon bombs with HNO_3 (sp, 65% v/v) at 60 ºC for 12 hours followed by 1h at 100 ºC, after which H_2O_2 (sp, 30%, v/v) was added and the digestion was completed at 80 ºC for 1 hour (Ferreira, 2002). The quantification was made by ICP-MS in a Thermo Elemental - X Series. The accuracy of the analytical procedures was verified through the analysis of certified reference materials, DORM-1 and 2 (dogfish muscle), TORT-1 and 2 (lobster hepatopancreas). Obtained and certified values (Table 2.3.1) were not statistically different ($p<0.05$). Detection limits were 0.010 and 0.096 μg g^{-1} (dry weight) for Hg, and Se, respectively. In all analysis, precision, expressed as relative standard deviation of three replicate samples were less than 8%.

For MeHg analysis a modified Westöö (1967) and Armstrong et al. (1999) methodology was used. Briefly, approximately 2 mL of Milli-Q water and 3 mL of 6M KOH solution were added to 200 mg of dried sample. The mixture was shaken for 2 hours and after 3 mL of 6M HCl and 4 mL of a $KBr/CuSO_4$ (3:1) solution was added. After 10 minutes of shaking, 5 mL of dichloromethane (DCM) was then added, the mixture centrifuged and finally the organic phase separated. A slight sulphide solution (≈ 0.06 mM) was used to extract MeHg from the organic phase and then MeHg was back extracted to DCM. Methyl-mercury in DCM was quantified by GC-AFS in an Agilent chromatograph coupled with a pyrolysis unit and a PSA Hg fluorescence detector. The possible MeHg artifact formation were evaluated by spiking several samples with Hg(II) and MeHg standard solutions of different concentrations. Recoveries varied between 92 and 103% and no artifact MeHg formation was observed. For all the analysis, precision expressed as the relative standard deviation of 3 replicate samples, was less than 2% ($p<0.05$). International certified standards DORM-2 and TORT-2 were used to ensure the accuracy of the procedure. Methyl-mercury concentrations were consistently within the ranges of certified values (Table 2.3.1). All concentrations are given as ranges and medians expressed microgram per gram of dry weight tissue (μg g^{-1}, dry weight).

Table 2.3.1 – Mercury, MeHg and Se concentrations (μg g^{-1}, dry wt) of dogfish muscle (DORM-1 and DORM-2) and lobster hepatopancreas (TORT-1 and TORT-2) (NRCC) determined in the present study and certified values.

Standard	Hg	MeHg	Se
		(μg g^{-1})	
DORM-1			
Present study	0.791±0.007	-	1.48±0.18
Certified	0.789±0.074	-	1.62±0.12
DORM-2			
Present study	4.67±0.11	4.51±0.42	1.44±0.14
Certified	4.64±0.26	4.47±0.32	1.40±0.09
TORT-1			
Present study	0.31±0.05	0.121±0.008	6.70±0.42
Certified	0.33±0.06	0.128±0.014	6.88±0.47
TORT-2			
Present study	0.30±0.04	0.155±0.006	5.46±0.42
Certified	0.27±0.06	0.152±0.013	5.63±0.67

Statistical analysis

Prior to statistical analysis, metal concentrations and biological parameters were tested for normality and equality of variances. The Mann-Whitney U and Kruskal-Wallis tests were applied to all data in order to detect differences between metal concentrations and biological parameters and tissues. The significance used for statistical analyses was $p<0.05$. The statistical analyses were performed using the STATISTICA 6.0 Statistical Software System.

Results

Biological data

Proportions female:male in the sampled octopus from Matosinhos, Cascais and Olhão were 6:6, 5:6 and 7:3, respectively. Both size and weight of those individuals varied over similar ranges: 125-163 mm and 578-1177 g in Matosinhos, 120-160 mm and 805-1570 g in Cascais and, 113-158 mm and 814-1406 g in Olhão. Size, weight and sex were not significantly different in specimens from the three areas. Differences of these variables between sampling periods have also no statistical validity.

Metal concentrations in digestive gland and mantle

The absence of relationships between metal concentrations and abovementioned biological parameters allow the treatment of results of both digestive gland and mantle from each site independently of the size/weight and gender of the individuals. Figure 2.3.2 shows the median, the percentile 25% and 75%, minimum and maximum of Hg, MeHg and Se concentrations, as well as the percentage of MeHg, in digestive gland and mantle of *O. vulgaris* captured in the three areas. Levels (µg g^{-1}) of total Hg and MeHg in digestive gland (0.36-7.4 and 0.18-5.0, respectively) were higher than in mantle (0.13-0.76 and 0.11-0.75, respectively). Selenium also presented higher levels (µg g^{-1}) in digestive gland (6.5-28) than in mantle (1.3-2.6). The significance of the elevated concentrations of Hg, MeHg and Se in digestive gland was confirmed by the Mann-Whitney U test. The percentage of MeHg in mantle (70-99%) was significantly higher than in digestive gland (41-96%).

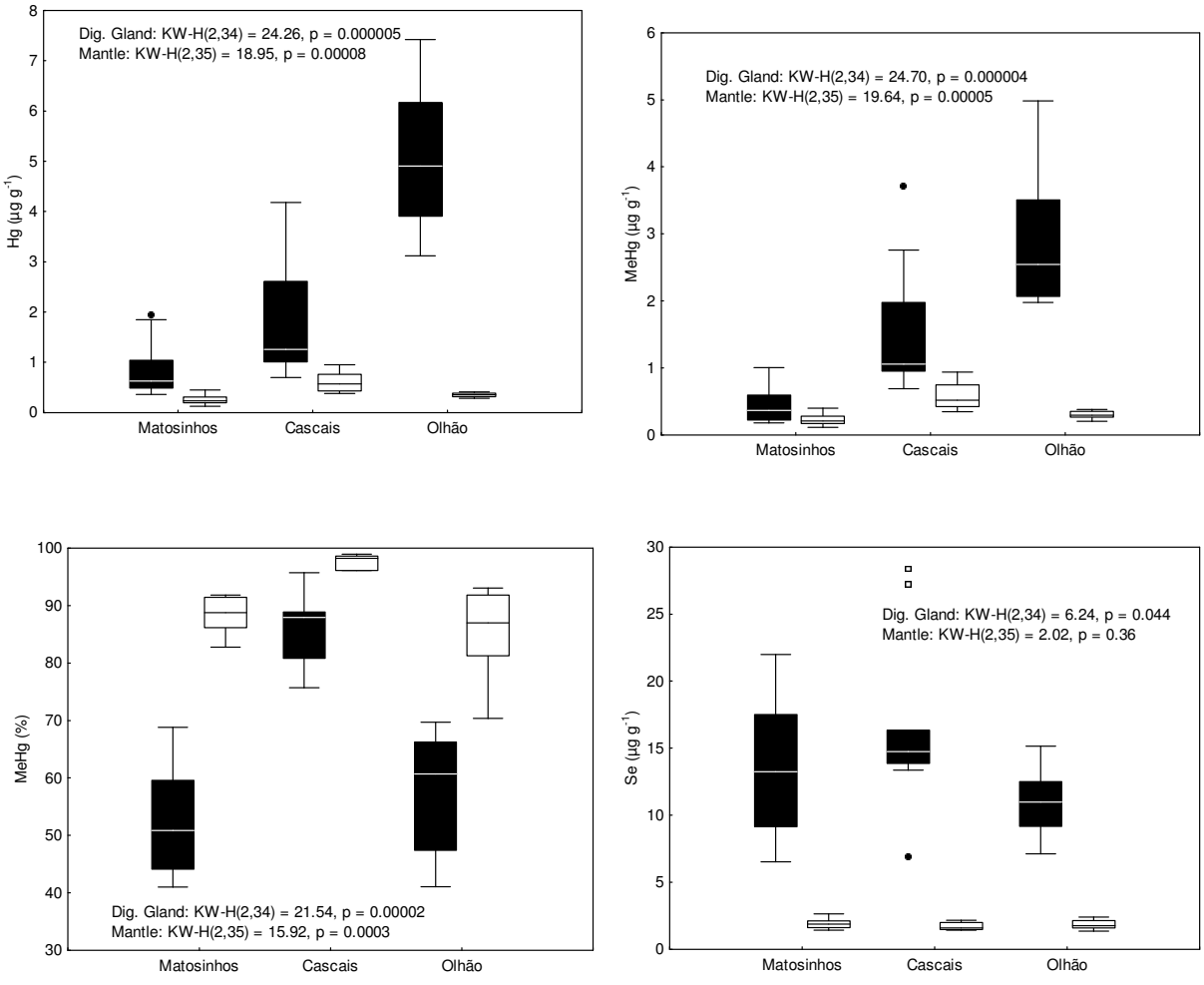

Figure 2.3.2 - Median, 25 and 75% percentile, minimum and maximum, and the extreme values (□) and outliers (•), of Hg, MeHg and Se concentrations (µg g^{-1}, dry weight) and MeHg (%) in the digestive gland (black boxes) and mantle (white boxes) of common octopus, *O. vulgaris* from the three areas of capture.

Differences between areas of capture

Digestive gland showed significantly higher levels of Hg and MeHg in specimens from Olhão than from Matosinhos and Cascais. Conversely, mantle exhibited significantly enhancements of Hg and MeHg in individuals from Cascais. The two analysed tissues showed also significantly elevated percentage of MeHg in specimens from Cascais. No significant differences of Se concentrations were found in digestive gland and mantle of individuals from the three areas of capture. Mercury and MeHg concentrations presented narrower intervals in mantle of specimens from Olhão (elevated values) than from Matosinhos and Cascais.

Discussion

Effect of size and gender on Hg accumulation

The results obtained with octopus captured in the three areas pointed to the lack of relationships between metal concentrations in digestive gland or mantle and size/weight or gender. Although Hg accumulated in fish in general increases with age (e.g. Joiris et al., 1999), the literature reflects a lack of consensus on the effect of these variables to Hg accumulation in cephalopods. Whereas some studies showed similar concentrations in small and large individuals (Barghigiani et al., 1991; Raimundo et al., 2004; Seixas et al., 2005a), others indicated correlation between accumulated levels and size of cephalopods (Monteiro et al., 1992; Rossi et al., 1993; Storelli and Marcotrigiano, 1999). No relations were found between Se concentrations in arms of the common octopus and body length (Seixas et al., 2005b). A variety of situations were reported with respect to gender: no differences for Hg and Se between male and female (Monteiro et al., 1992; Raimundo et al., 2004; Seixas et al., 2005a, b), decrease of Hg uptake in mature females of *E. cirrhosa* (Rossi et al., 1993), higher levels in females of *L. forbesi* (Bustamante et al., 2006) and lower levels in females (Pierce et al., 2008). The discrepancy of these observations resulted probably from the more prominent effect of different factors, such as food availability (i.e., quality and quantity of food) and growth rates (which may be affected by temperature) (Villanueva et al., 2002), on the metal accumulation in cephalopods. The marked differences of accumulated Hg in individuals from the three areas led us to hypothesise the relevance of geographical differences that could be associated with Hg availability in food chain or water.

Comparison of metal levels with the literature

Table 2.3.2 compares concentrations of Hg and Se of the current study with those reported in the literature for *O. vulgaris*. Levels of Hg in digestive gland and mantle of specimens from Matosinhos and Cascais are comparable to values reported in previous studies for the Portuguese coast (Raimundo et al., 2004; Seixas et al., 2005a), Azores Islands (Monteiro et al., 1992) and North Eastern Atlantic waters (Bustamante et al., 2006). Digestive gland of octopus from Olhão exceeded the levels registered in this tissue of individuals from the Adriatic Sea (Storelli et al., 2006) that is known to receive emission of Hg from mining areas of the region (Faganeli et al., 2003). Selenium also ranged within the same concentration interval reported in a previous work on octopus captured in the Portuguese coast (Seixas et

al., 2005b). To the best of our knowledge the current work reports for the first time levels of MeHg in cephalopods.

The elevated Hg concentrations in digestive gland relatively to mantle are in line with previous works. For example, Bustamante et al. (2006) found that total Hg concentrations in digestive gland of twenty species of squids, cuttlefishes and octopuses from the North Eastern Atlantic waters were higher than in remaining tissues. A similar partitioning was observed for *O. salutii* and *I. coindeti* and *L. vulgaris*, *O. vulgaris*, *E. cirrhosa*, *E. moschata*, *S. orbignyana*, *S. officinalis* from the Adriatic Sea (Storelli and Marcotrigiano, 1999; Storelli et al., 2006) and *O. vulgaris* from the Portuguese coast (Raimundo et al., 2004; Seixas et al., 2005a). This preferential accumulation in the digestive gland reflects its ability to absorption, assimilation and storage of metals (e.g. Miramand and Guary, 1980; Finger and Smith, 1987; Bustamante et al., 2002; Raimundo and Vale, 2008). However, the digestive gland has been pointed out as an important organ of mercury detoxification (Bustamante et al., 2006).

Table 2.3.2 – Comparison of Hg and Se levels (μg g^{-1}, dry weight) in the digestive gland and mantle of *O. vulgaris* from Portuguese coast (Matosinhos, Cascais and Olhão) with values in the literature.

Organ/tissue of *Octopus vulgaris*	Hg	Se	Authors
	μg g^{-1}		
Digestive Gland			
Portuguese coast – Matosinhos	0.86±0.54	14±5.1	Present study
Portuguese coast – Cascais	1.8±1.1	17±6.4	Present study
Portuguese coast – Olhão	5.1±1.5	11±2.5	Present study
North Eastern Atlantic waters	1.1±0.2	-	Bustamante et al. (2006)
Adriatic Sea	2.7±1.2*	-	Storelli et al. (2006)
Portuguese coast – Viana	0.58±0.08	-	Seixas et al. (2005a)
Portuguese coast – Cascais	3.4±2.6	-	Seixas et al. (2005a)
Portuguese coast	2.6±4.3	-	Raimundo et al. (2004)
Mantle			
Portuguese coast – Matosinhos	0.28±0.14	1.9±0.4	Present study
Portuguese coast – Cascais	0.60±0.20	1.7±0.3	Present study
Portuguese coast – Olhão	0.35±0.04	1.8±0.3	Present study
Adriatic Sea	1.5±1.0[a]	-	Storelli et al. (2006)
Portuguese coast – Viana	0.27±0.04	-	Seixas et al. (2005a)
Portuguese coast – Cascais	0.48±0.16	-	Seixas et al. (2005a)
Portuguese coast – Viana	-	1.1±0.4	Seixas et al. (2005b)
Portuguese coast – Cascais	-	1.5±0.2	Seixas et al. (2005b)
Portuguese coast	0.31±0.12	-	Raimundo et al. (2004)
Azores	0.38±0.35[b]	-	Monteiro et al. (1992)

[a] Calculated from wet weight (flesh).

[b] Calculated from wet weight (Muscle).

Elevated concentration of Hg in octopus from SE Portuguese coast

Miramand and Bentley (1992) proposed that the ratio between digestive gland and mantle concentrations gives an estimation of the contamination degree in cephalopods. The calculation of this ratio for Hg data indicates that individuals from Olhão are moderately contaminated (10<ratio<50), while specimens from Matosinhos and Cascais are low contaminated (ratio<10). The moderate Hg contamination in specimens from the SE Portuguese coast (Olhão) could be related to the influence of large sulphide deposits of the Iberian Pyrite Belt in the southern region of the Peninsula (Leistel et al., 1998). This geological feature has been shown to affect Hg concentrations in water and suspended particulate matter of the Gulf of Cadiz (Cossa et al., 2001). The water circulation in the eastern shelf of the South coast of Portugal, which is characterised by a cyclonic cell (Garcia Lafuente and Ruizet, 2006; Relvas et al., 2007) may augment the Hg availability to local food web. Octopus being a top predator (Mangold, 1983) tends thus to amplify the Hg signal in the region. Mediterranean Sea imports inorganic-Hg through the Gibraltar straits and exports it to the Atlantic Ocean partially as methylated species (Cossa et al., 1997). However, the accumulated Hg in near-shore octopus from SE coast of Portugal appears to be inadequate to trace the influence of this source. The low proportion of MeHg in digestive gland of octopus from Olhão (figure 2) reinforces the supposition of minor influence of the Mediterranean outflow on accumulated mercury in octopus from Olhão region.

Relationships between levels in digestive gland and mantle

Relationships were examined by plotting Hg and MeHg levels in mantle against values in digestive gland (Figure 2.3.3). For the less contaminated samples (Matosinhos and Cascais), where differences between tissues were less marked, a tendency to a proportional increase in the two organs was obtained. This proportionality suggests that as MeHg enters the digestive gland (via food) is partially transported and storage in mantle. In fish, it is proposed that the high affinity of MeHg for thiol groups containing amino acids (e.g. cysteine) facilitates the transport to muscle tissues (Leaner and Mason, 2004), where it may be firmly bond by carbon-mercury and sulphydryl linkages (Ruelas-Inzunza et al., 2003). A similar mechanism may be invoked to explain the high percentage of MeHg in octopus mantle. The better relationship between MeHg and Hg in mantle (R^2=0.99) with comparison with in digestive gland (R^2=0.88) may reflect that same affinity (Figure 2.3.4). The lower proportion of MeHg in digestive gland is in accordance with the lower retention of organic-Hg compounds in digestive gland found for various species of cephalopods (Bustamante et al., 2006). The transport of MeHg to mantle was particularly efficient in specimens from Cascais, reaching 98% of the accumulated Hg. A possible explanation is its availability, either in food web or in abiotic compartments. In favour of this hypothesis is the higher proportion of MeHg in digestive gland of specimens from Cascais (88%) with respect to individuals from Matosinhos (51%) and Olhão (61%). Indeed, food type in addition to Hg partitioning within cells play a major role on the assimilation efficiency of MeHg (Mason et al., 1995; Mason et al., 2004). Although

previous studies showed different stomach contents in octopus from the three studied areas (Rosa et al., 2004), the lack of information on Hg concentration in diet limits further discussion.

Figure 2.3.3 also showed that MeHg in mantle of octopus from Olhão (the most contaminated samples) is not proportional to increases found in digestive gland. The narrow range observed in mantle indicates a less efficient transfer of this compound from the digestive gland.

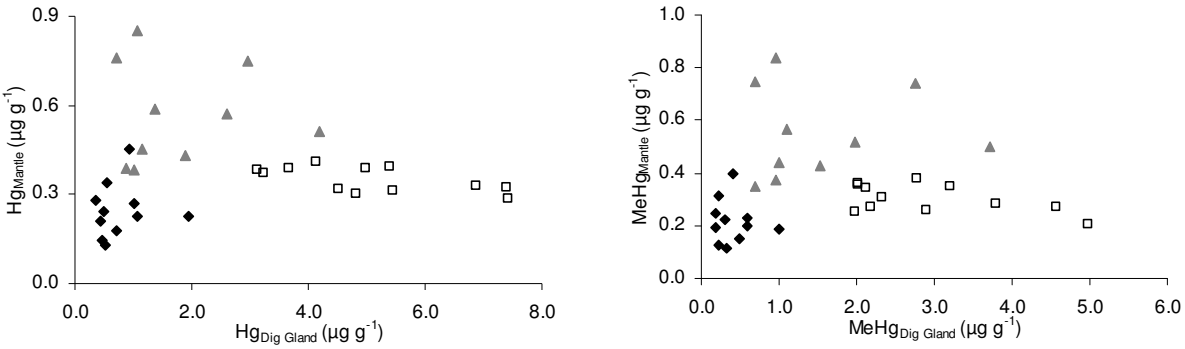

Figure 2.3.3 – Relationships between concentrations of Hg and MeHg (µg g^{-1}, dry weight) in mantle and digestive gland of *O. vulgaris* from Matosinhos (♦), Cascais (▲) and Olhão (□).

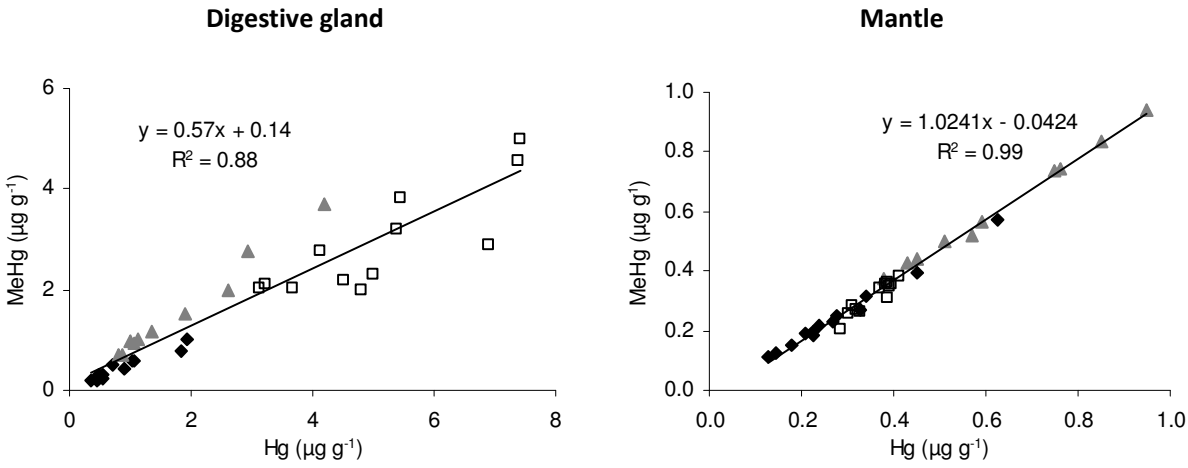

Figure 2.3.4 – Relationships between concentrations of Hg and MeHg (µg g^{-1}, dry weight) for the digestive gland and mantle of *O. vulgaris* from Matosinhos (♦), Cascais (▲) and Olhão (□).

Selenium and Mercury

Yang et al. (2008) proposed the involvement of Se in the demethylation of MeHg to form inorganic and less toxic Hg compounds. This involvement is usually scrutinised by the equimolarity between total Hg and Se in biological organs (Nigro and Leonzio, 1996). The Se:Hg molar ratio calculated for the digestive gland of octopus showed that Se was present in a surplus to Hg in all samples from

Matosinhos and Cascais: median (and range) of the Se:Hg ratio reached 32 (17-136) and 30 (6-68), respectively. Conversely, the median (and range) of the ratio in digestive gland of octopus from Olhão was 5.4 (4-12). This proximity to the unit suggests a higher probability of the activation of a demethylation mechanism in digestive glands containing higher MeHg concentrations, CH_3Hg^+ being decomposed and HgSe formed. This hypothesis is in line with the partial demethylation of the organic forms of mercury in digestive gland of various cephalopods species proposed by Bustamante et al. (2006). A recent study by Yang et al. (2010), suggest that a threshold concentration of Se in fish body parts must be reached before a clear protective role of Se against Hg assimilation become noticeable. The conjugation of higher levels of Hg in digestive gland of octopus from Olhão, lower Se:Hg ratio, and lower and narrower concentration interval of MeHg in mantle suggests that the threshold concentration was exceeded in digestive gland. Because Se concentration was similar in digestive gland of specimens from the three study areas, one may assume that protective role of Se against Hg assimilation was active in all specimens. However, demethylation was more noticeable in Olhão.

Octopus as a source of Hg in human consumption

Cephalopods are an important food resource being consumed in large quantities in several countries world wide (Amaratunga, 1983). In general, mantle of octopus, which is the commercial item, contain low Hg concentrations, generally below the safety limit established by the European Commission (0.5 μg g^{-1}, ww of Hg, Journal of EU Communities 2006, EC rule no. 1881/2006). All the specimens presented levels below those limits. According to the joint FAO/WHO expert committee the Provisional Tolerable Weekly Intake (PTWI) recommended for Hg and MeHg is approximately 5 and 1.6 μg kg^{-1} body weight per week, respectively (WHO, 2003). To exceed Hg values it would be necessary to ingest 212 g of mantle of individuals from Cascais, 1204 g of specimens captured in Olhão and 1529 g of individuals from Matosinhos per week. However, since most of the Hg found in the muscle tissue is MeHg (70-99%) the amount of octopus mantle that could be consumed would reduce 2 times to around 80 g (Cascais), 447 g (Olhão) and 550 g (Matosinhos).

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Chapter 3

Sub-cellular responses to elemental concentrations

Context

The preceding chapter described the metal levels in various tissues of octopus and the responses of tissues to the environmental availability.

Metals in excess are potentially toxic and should be removed in order to protect important biological molecules. In that sense, detoxifying mechanisms must be activated to prevent toxic substances from affecting metabolism or damaging sensitive structures within cells.

Summary

This chapter describes the concentrations of V, Co, Cu, Zn, As, Cd and Pb and their sub-cellular distributions (granules, mitochondria, lysosomes plus microsomes, heat-denaturable and heat-stable proteins) in digestive gland, kidney and gills of the common octopus, *Octopus vulgaris* collected in areas with contrasting levels of contamination. The association with proteins of different molecular weight and the presence of metallothioneins were also examined. The associations with the different fractions are presented herein.

Chapter 3.1

Sub-cellular partitioning of Zn, Cu, Cd and Pb in the digestive gland of native *Octopus vulgaris* exposed to different metal concentrations (Portugal)

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.

Abstract

Concentrations of Zn, Cu, Cd and Pb and their sub-cellular distributions were determined in composite samples of digestive glands of the common octopus, *Octopus vulgaris* caught from two areas of the Portuguese coast characterised by contrasting metal contamination. A minor content of Zn (1%), Cu (2%), Cd (6%) and Pb (7%) were found in the insoluble fraction, consisting of nuclei, mitochondria, lysosomes and microsome operationally separated from the whole digestive gland through a sequential centrifugation. A tendency for linear relationships between metal concentrations in nuclei, mitochondria, lysosomes and whole digestive gland was observed. These relationships suggest that despite low metal content organelles responded to the increasing accumulated metals, which means that detoxifying mechanism in cytosol was incomplete. Poorer correlations between microsome and whole digestive gland did not point to metal toxicity in the analysed compartments. However, the high accumulated Cd indicated that *O. vulgaris* is an important vehicle of this element to its predators in the coastal environment.

Introduction

Cephalopods are known for their ability to accumulate high levels of essential and non-essential elements in the digestive gland, which plays a key function in the digestive process (Martin and Flegal, 1975; Miramand and Guary, 1980; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante et al., 1998a, b; 2000).

Metal partitioning among tissues are highly dependent on biochemical processes occurring within cells (Langston et al., 1998). Following absorption into the circulatory system, metals are transported to specific organs where they are utilized in normal metabolism, eliminated or sequestered. When a metal is incorporated into a molecule (e.g. hemocyanin or metalloenzymes) it enters the routine metabolic pathways. When trace metals are in excess amounts or are toxic, they are complexed and removed from interaction with other biologically important molecules, either by sequestration or excretion (Engel and Brouwer, 1984). The binding of an “inappropriate” metal to a metal-sensitive site, like organelles and enzymes, is often associated with detoxification mechanisms (Simkiss and Taylor, 1982; Phillips and Rainbow, 1989; Bustamante et al., 2002) and could be an indicator of metal–induced stress (Wallace et al., 2003; Campbell et al., 2005). Manifestations of sub-lethal toxicity can coincide with changes in subcellular partitioning, particularly in cases where there is saturation of certain metal detoxification systems (Sanders et al., 1983; Wallace et al., 2003). The involvement of the subcellular compartments in metal sequestration has been investigated in laboratory experiments with molluscs (e.g. Bebianno and Langston, 1992; Hylland et al., 1994; Roméo and Gnassi-Baelli, 1995). Less data is available for metal sub-cellular partitioning in cephalopods (Tanaka et al., 1983; Finger and Smith, 1987; Bustamante et al., 2002) and these works consider mostly the partition between soluble and insoluble fractions.

Various works reported the metal concentrations in tissues of *Octopus vulgaris* captured along the Portuguese coast (Raimundo et al., 2004, 2005; Napoleão et al., 2005; Seixas et al., 2005a, b). The

comparison of accumulated metals in specimens captured along the coast was important because metal availability in water and seston differ considerably between NW and S Portuguese coast (Caetano and Vale, 2003). However, those works only reported concentrations at tissue level and did not test the hypothesis of the organelles response and the relationships with detoxifying mechanisms in cytosol (Bonneris et al., 2005). This study presents the Zn, Cu, Cd and Pb concentrations in digestive glands of the common octopus, *O. vulgaris* captured in NW and S Portuguese coast, and their sub-cellular distributions in nuclei, mitochondria, lysosomes and microsomes operational insoluble fractions.

Material and Methods

Samples

Octopi, *Octopus vulgaris*, were captured in March and October 2005 and February 2006 from the areas of Matosinhos (M), and Olhão (O), located in the northwest and south coast of Portugal (Figure 3.1.1).

Figure 3.1.1 – Location of the two sampling sites of *O. vulgaris* in the Portuguese coast: Matosinhos and Olhão.

The specimens were weighted, measured and digestive gland removed and frozen at -80ºC. Prior to the preparation of composite samples Zn, Cu, Cd and Pb were determined in whole digestive gland of single individuals. The digestive glands of specimens from each sampling period and area presenting metal concentrations that varied within ±20% were considered for the preparation of composite samples. Six composite samples of digestive gland were prepared from Matosinhos and seven from Olhão. The ranges of weight and mantle length of individuals which digestive glands were incorporated in composite samples are presented in Table 3.1.1. Composite samples were homogenised at a dilution of 1:3 (wet weight:volume of buffer) in an ice bucket. The buffer consisted of Tris-HCl (10 mM, pH 7.4, and 0.15M

NaCl) and 1mM PMSF (phenylmethylsulfonylfluoride, as protease inhibitor). The homogenation was completed in short periods of time (<5 min.) and low speed (< 6000 rpm) to minimize organelle breakage.

Table 3.1.1 – Number of individuals (n), and ranges of weight (g) and length (mm) of specimens included in the composite samples of digestive gland of *Octopus vulgaris* from Matosinhos and Olhão; three sampling periods were considered.

Sample	Sampling Periods	n	Weight (g)	Length (mm)
	Matosinhos			
M1	March.05	5	796 - 1433	125 - 170
M2	October.05	2	847- 956	125 - 145
M3		3	852 - 914	130 - 147
M4		2	1057 - 1177	135 - 140
M5	February.06	3	1021 - 1049	125 - 155
M6		1	853	125
	Olhão			
O1	March.05	2	850 - 1520	125 - 170
O2		2	935 - 1105	140 - 150
O3		4	827 - 1520	135 - 135
O4	October.05	3	902 - 1111	154 - 157
O5		2	941 - 953	160 - 165
O6	February.06	3	1068 - 1406	135 - 153
O7		3	1001 - 1084	134 - 158

Sub-cellular fractionation

For subcellular analyses, a sub-sample of each homogenate was transferred to centrifuge tubes and subjected to differential fractionation. The procedure adapted from Campbell et al. (2005) is schematically the following: the homogenate was first fractioned by centrifugation at 700 x g for 15 min at 4ºC to separate the nucleus; the supernatant was further centrifuged at 9 000 x g for 20 min at 4ºC to separate the mitochondrial fraction; the lysosome and microsomal fractions were obtained by centrifuging the supernatant at 30 000 x g for 25 min, and at 100 000 x g for 40 min at 4ºC, respectively. The four fractions obtained by the centrifugation procedure were lyophilized for metal analyses.

Metal analyses

Zinc, Cu, Cd and Pb were analysed in lyophilised samples of individual digestive glands, composite samples of whole digestive glands and pellets after digestion with a mixture of HNO_3 (sp, 65% v/v) and H_2O_2 (sp, 30% v/v) at 60 ºC for 12 hours and 100 ºC for 1 hour according to the method described in Ferreira et al. (1990). All lab ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Metal concentrations were determined by flame atomic absorption

spectrometry (Perkin Elmer AAnalyst 100) or graphite furnace atomic absorption spectrometry (Perkin Elmer, Zeeman 4110ZL). The accuracy of these analytical methods was assessed by the analysis of international certificate standards (TORT-1 and TORT-2). Measured and certified values did not differ significantly ($p<0.05$) (Table 3.1.2).

Table 3.1.2 - Zinc, Cu, Cd and Pb concentrations (μg g^{-1}, dry wt) of lobster hepatopancreas certificate standards (TORT-1 and TORT-2) (NRCC) determined in the present study and certified values.

Standard	Zn	Cu	Cd	Pb
	μg g^{-1}			
TORT-1				
Present study	168±13	379±24	25±2.9	11±2.7
Certified	177±10	439±22	26.3±2.1	10.4±2
TORT-2				
Present study	173±11	96±5.0	29±1.7	0.33±0.073
Certified	180±6	106±10	26.7±0.6	0.35±0.13

Statistical analyses

Prior to statistical analyses, metal concentrations and biological parameters were tested for normality and equality of variances. The Mann-Whitney U test was used to evaluate the existing differences between metal concentrations in the digestive glands and pellets, and to compare the weight and mantle length of individuals from Matosinhos and Olhão areas. The significance for statistical analyses used was always $\alpha<0.05$. The statistical analyses were performed using the SATISTICA 6.0 Statistical Software System.

Results

The medians and ranges of Zn, Cu, Cd and Pb concentrations in the sub-cellular fractions and the whole digestive gland of octopus caught in March and October 2005 and in February 2006 in the Matosinhos and Olhão areas are given in Table 3.1.3. The concentrations of each determined metal varied within broad ranges in the whole tissue and organelles of the composite samples from the two sampling areas.

Table 3.1.3 – Median and ranges of Zn, Cu, Cd and Pb concentration (μg g^{-1}, dry weight) in whole digestive gland and their insoluble fractions (nuclei, mitochondria, lysosomes and microsomes) of common octopus (n=6 Matosinhos; n=7 Olhão).

	Zn	Cu	Cd	Pb
	μg g^{-1}			
Matosinhos				
Whole tissue	850 (410 – 2040)	860 (640 – 1490)	150 (57 – 250)	2.4 (1.5 - 4.1)
Nuclei	1070 (570 – 1660)	1100 (90 – 1210)	120 (28 – 500)	5.4 (4.5 – 22)
Mitochondria	1300 (390 – 18180)	740 (520 – 3260)	150 (36 – 360)	6.4 (1.9 – 31)
Lysosomes	630 (220 – 10110)	620 (220 – 13020)	100 (100 – 290)	3.2 (1.4 – 5.7)
Microsomes	1750 (1080 – 2250)	650 (600 – 2890)	230 (190 – 1080)	6.4 (1.5 – 25)
Olhão				
Whole tissue	1840 (740 – 2870)	1390 (1120 – 1600)	15 (10 – 30)	4.8 (3.0 – 7.2)
Nuclei	3880 (1230 – 1660)	1160 (310 – 1670)	22 (20 – 210)	8.6 (2.7 – 16)
Mitochondria	2600 (990 – 18230)	890 (410 – 1280)	21 (17 – 45)	12 (4.7 – 21)
Lysosomes	1960 (630 – 6420)	910 (440 – 2000)	25 (17 – 42)	10 (2.4 – 26)
Microsomes	1900 (1150 – 17060)	1310 (720 – 1570)	21 (15 – 140)	7.0 (2.5 – 34)

Whole digestive gland

The essential elements Zn and Cu were the most abundant determined metals in the composite samples of the whole digestive gland, varying between 410 and 2870 μg g^{-1} and from 640 to 1600 μg g^{-1}, respectively. Levels of Cd and Pb were one to three orders of magnitude below. Accumulated metals in the samples of the three sampling periods were not statistically different ($p<0.05$). Cadmium showed significantly ($p<0.05$) higher values in digestive glands of individuals from Matosinhos (57 to 250 μg g^{-1}) than from Olhão (10 to 30 μg g^{-1}). Lead presented a narrower concentration range (1.5 to 7.2 μg g^{-1}) with significantly enhanced levels in digestive glands of samples from Olhão. The length and weight of octopus from Matosinhos and Olhão areas did not differ significantly ($p<0.05$) and consequently differences on accumulated Cd and Pb can not be attributed to those allometric parameters. Although no significant

differences were found for Zn and Cu, with those parameters, concentrations in the whole tissue of specimens from Olhão were in general higher than from Matosinhos.

Insoluble fraction

The metal concentrations did not varied significantly between the four separated organelles (nuclei, mitochondria, lysosomes and microsomes) of the digestive gland homogenates, in specimens from both Matosinhos and Olhão (Table 3.1.3). However, accumulated levels varied between the two areas. Cadmium was significantly ($p<0.05$) higher in mitochondrial, lysosomal and microsomal fractions of samples from Matosinhos. Levels of the other determined metals were significantly higher in Olhão for nuclei and lysosomes (Zn), microsomes (Cu) and mitochondria and lysosomes (Pb). At each site, metal concentrations in organelles did not varied significantly between the three sampling periods. By comparing the metal content amounted in the four organelles and in whole tissue one may estimate that only 1% (Zn), 2% (Cu), 6% (Cd) to 7% (Pb) of total determined metals are in the insoluble fraction.

Discussion

Table 3.1.4 compares the metal concentrations registered in the whole digestive gland of *O. vulgaris* from Matosinhos and Olhão with values reported in the literature for cephalopod species. Zinc levels ranged within the interval referred for the same species in the Mediterranean Sea (Miramand and Guary, 1980) and they were lower than maximum concentrations measured in previous works along the Portuguese coast (Raimundo et al., 2004; 2005; Napoleão et al., 2005). Copper levels were also comparable to previous data (Raimundo et al., 2004; 2005; Napoleão et al., 2005) and slightly lower than values of Mediterranean Sea (Miramand and Guary, 1980). The comparison with other cephalopod species from various regions (Martin and Flegal, 1975; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante et al., 1998a) indicates similar or slightly higher values of Zn and an inter-specific variability of Cu. The involvement of these elements in a number of metabolic functions, such as in metal-dependant enzymes (Bustamante et al., 2000; Craig and Overnell, 2003), may explain their high concentrations. However, enrichment at certain component of the food web may result in broader concentration intervals in the digestive gland of cephalopods.

Table 3.1.4 – Comparison of Zn, Cu, Cd and Pb levels (μg g^{-1}, dry weight) in the digestive gland of *O. vulgaris* from Matosinhos and Olhão with cephalopod data from the literature.

Species	Zn	Cu	Cd	Pb	Authors
	μg g^{-1}				
Octopus vulgaris	410-2873	639-1597	10-252	1.5-7.2	Present study
Octopus vulgaris	200-14721	139-3140	19-761	0.037-44	Raimundo et al. (2004)
Octopus vulgaris	198-14721	137-1465	20-269		Raimundo et al. (2005)
Octopus vulgaris	1463±726	1768±1010		6.9±3.2	Napoleão et al. (2005)
Octopus vulgaris	1450±400	2500±700	50±10	-	Miramand and Guary (1980)
Sepia officinalis	571±47	315±3	13±0.35	-	Miramand and Bentley (1992)
Sepia officinalis	220-5678	68-5054	10-557	-	Raimundo et al. (2005)
Loligo opalescens	247±131	5350±3210	85±52	-	Miramand and Flegal (1975)
Nototodarus gouldi	830±355	363±238	33±30	-	Finger and Smith (1987)
Eledone cirrhosa	646±86	456±11	24±1.8	-	Miramand and Bentley (1992)
Benthoctopus thielei	416	42	215	-	Bustamante et al. (1998a)
Graneledone sp.	102	1092	369	-	Bustamante et al. (1998a)
Nautilus macromphalus	672±208	106±46	45±13	-	Bustamante et al. (2000)

Cadmium concentrations in the whole digestive gland of *O. vulgaris* were similar to previous values reported for the same species captured in the Portuguese coast (Raimundo et al., 2004; 2005), however the maximum values exceeded largely the levels found in the same species from the Mediterranean Sea (Miramand and Guary, 1980), in other octopus species (*Eledone cirrhosa*, *Graneledone sp.*), and in other cephalopods (*Sepia officinalis, Nototodarus gouldi, Loligo opalescens, Ommastrephes bartrani* and *Nautilus macromphalus*) (Martin and Flegal, 1975; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante *et al*., 1998a; Bustamante et al., 2000). Levels of Cd registered in this work was only comparable to the ones reported for *Benthoctopus thielei* and *Graneledone* sp. (Bustamante et al., 1998a), and *Sepia officinalis* (Raimundo et al., 2005). Lead concentrations varied in a narrow range and comparable to values obtained for individuals captured in the Portuguese coast (Napoleão et al., 2005). Higher values found in digestive gland of *O. vulgaris* were interpreted as due to pollution sources (Raimundo et al., 2004).

The small contribution (<7%) of the insoluble fractions to the total Zn, Cu, Cd and Pb content in the digestive gland indicates that the large majority of these elements are trapped in the cytosolic proteins of *O. vulgaris*. Molluscs are known to have a number of subcellular systems for accumulation, regulation and immobilizing of metals during phases of excess (Langston et al., 1998). High retention of metals in cytosol has been frequently reported. For example, approximately 78, 70 and 47% of Cu, Cd and

Zn, respectively, were found associated with the soluble fraction in the digestive gland of the squid *Nototodarus gouldi* (Finger and Smith, 1987). Bustamante et al. (2002) studied the partition of Cd in the cephalopods *Loligo vulgaris, Illex coindetii, Sepia officinalis, Sepia elegans, Sepia orbignyana, Todarodes sagittatus* and *Eledone cirrhosa*, and found that 42-86% of Cd was present in the soluble fraction, depending on species. In the digestive gland of the squid *T. pacificus*, only 26% of Cd was associated with the cytosolic fraction (Tanaka et al., 1983). The high association of Cd to cytosol was confirmed under laboratory conditions in gills and digestive gland of the Mediterranean clam *Ruditapes decussates* (Roméo and Gnassi-Baelli, 1995), in gills, intestine, head-food tissue and hepatopancreas of the dog whelk *Nassarius reticulates* (Hylland et al., 1994), and in the soft parts of *Mytillus galloprovincialis* (Bebianno and Langston, 1992). Although less data is available for Pb, approximately 50% was found in sea turtles liver (Anan et al., 2002), and 33% in digestive gland of the scallop, *Chlamys varia* (Bustamante and Miramand, 2005).

Although the metal partitioning between soluble and insoluble fractions of the digestive gland varies with the cephalopod species, such a small retention in the insoluble fractions as observed in *O. vulgaris* (1-7%) is rarely reported. The organelles are recognised to be sensitive to metal contamination and its examination may provide a better understanding of potential mechanisms of toxicity and tolerance (Wallace et al., 2003). The partition of metals in these subcellular fractions is related to the fact that storage takes place in compartments that are particularly rich in, or capable of synthesizing relatively large quantities of metal-binding ligands (Langston and Spence, 1995). An interestingly aspect of this work was to examine whether that sensitivity occured even when only small percentages of metal contents are retained in the insoluble fractions. Because organelles were separated operationally by differential centrifugation, one should admit that potential artefacts were possible, such as, breakage or clumping of particles, leakage of soluble constituents from organelles and overlap among subcellular fractions (Wallace et al., 2003), that may confound the interpretation of results. For example, the centrifugation used in this work did not separate granules from nuclei, which may lead to the presence of metals with high affinity to granules (Markich et al., 2001; Bonneris et al., 2005) in the first sequential fraction, designated in this work as nuclei fraction.

Figure 3.1.2 presents the relationships of Zn, Cu, Cd and Pb concentrations between each separated organelle and in the whole digestive gland of individuals captured at Matosinhos and Olhão areas. Zinc levels in nuclei and lysosomes were linearly correlated to the values registered in the whole digestive gland. A similar tendency was observed for mitochondria and microsome fractions. Copper in the four organelles tends to increase with levels found in whole digestive gland, although poorer correlations were obtained. The same trend was observed by Bonneris et al. (2005) that showed correlation between Cu levels in the mitochondrial fraction and in the digestive gland of the freshwater bivalve *Pyganodon grandis*. Mitochondria, lysosomes and microsomes fractions follow the increment of Cd in the whole tissue through linear relationships. Lysosomes are known to be involved in the accumulation of essential and non-essential elements by removing them from the cytosol (Bustamante et

al., 2002). They also accumulate cellular waste products which cannot be degraded, like metalloproteins (Dallinger, 1993). The accumulation in mitochondrial and microsome fractions is often associated with toxicity (Bonneris et al., 2005). Lead in nuclei, mitochondria and lysosomes fractions also presented linear relationships with total Pb concentrations.

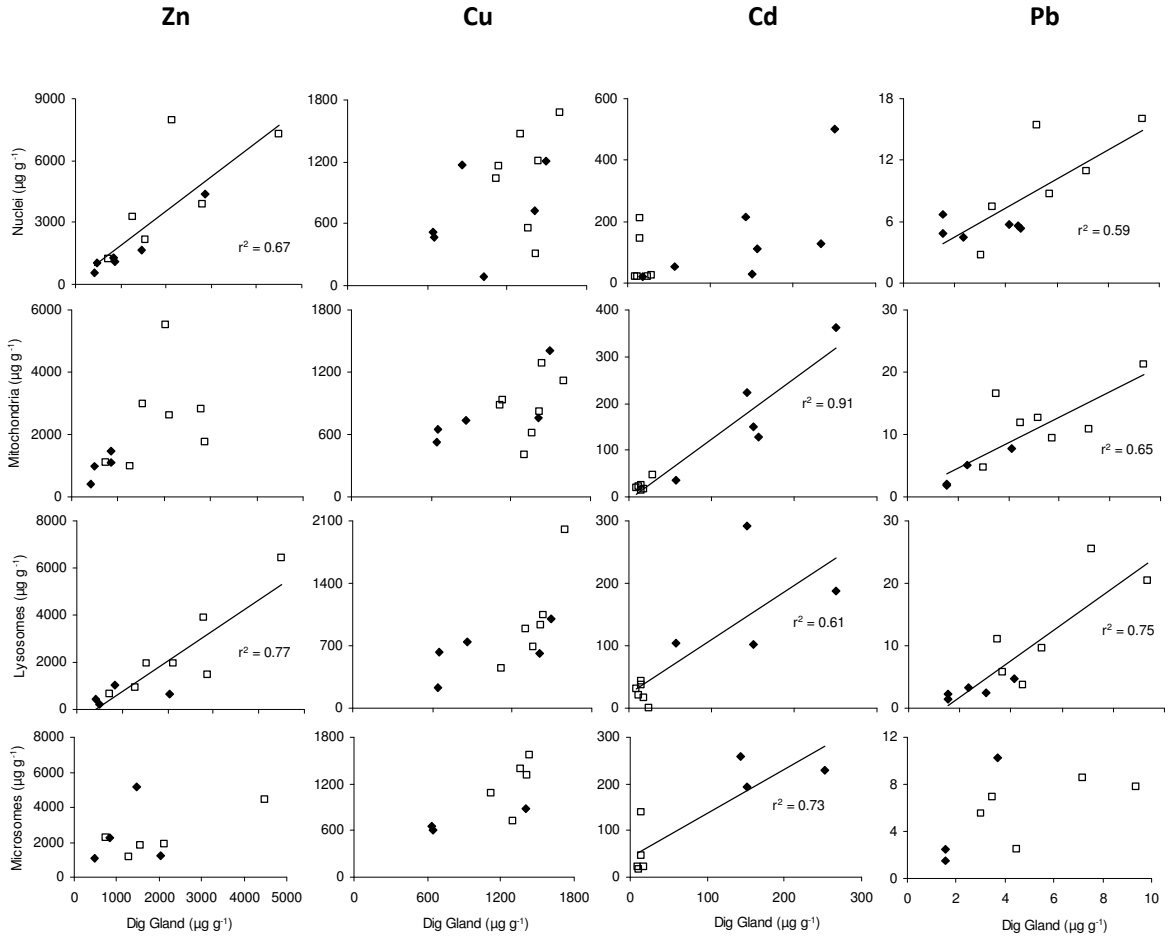

Figure 3.1.2 – Relationships between levels of Zn, Cu, Cd and Pb (µg g^{-1}, dry weight) in: nuclei, mitochondria, lysosomes and micrososmes and the whole digestive gland of *O. vulgaris* from Matosinhos (♦) and Olhão (□).

Despite the small content of metals in insoluble fractions of digestive glands of octopus, the tendency of metal concentrations in organelles to increase with the levels in the whole digestive gland indicates that animals had not successfully detoxified the non-essential metals. The enhanced levels of Zn, Cu and Pb in the nuclei fraction may be related to various factors because this is the most operational fraction containing cell membranes, intact cells, nuclei, granules, and other cellular components of unknown function (Bonneris et al., 2005). In particular granules are fairly ubiquitous in molluscs, though they may serve different functions within different cells in relation to the distribution of metals (Langston et al., 1998). The mitochondrial fraction is considered as a more metal-sensitive compartment (Bonneris

et al., 2005), because metals can bind to crucial enzymes and respiratory protein complexes. The increasing levels of Zn, Cu, Cd and Pb in mitochondria fraction with concentrations in the whole digestive gland are in line with the metal-sensitive nature of this compartment. Accumulated metals in this fraction reduce energy conversion efficiency and uncouple oxidative phosphorylation that causes oxidative damage (Di Giulio et al., 1995). Those biochemical responses were not determined in this study and consequently the possible associated damages can not be confirmed. The same trend was observed in lysosome fraction, which is known to accumulate metals from the cytosol of the digestive gland cells for eventual elimination (Bustamante et al., 2002; Viarengo et al., 1989; Langston et al., 1998). The obtained correlations with total concentration in digestive gland may be indicative that elimination was linearly related to the accumulation. It has been proposed that metals in the microsome fraction indicate the presence of fragmented endoplasmic reticulum, which is generally responsible for synthesis and transport of proteins (Jarosch et al., 2002; Bonneris et al., 2005). The elevated metal concentrations in this fraction could point to toxicity. However, analogous levels of metals among the four organelles and poorer correlations of metal concentrations between the microsome fraction and the whole tissue were not symptomatic of high toxicity condition in the octopus.

Despite the lack of evidence of metal toxicity in the analyzed organelles, the high accumulated Cd in the digestive gland point to *O. vulgaris* as an important vehicle of this element to its predators in the coastal environment. This supposition is in agreement with the work of Bustamante et al. (1998b) that proposed cephalopods as a vector for the transfer of Cd to top marine predators.

Acknowledgments

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Chapter 3.2

Sub-cellular partitioning of trace elements in digestive gland, kidney and gills of native *Octopus vulgaris* (Portugal)

Raimundo, J., Vale, C., Moura, I. (submitted). Sub-cellular partitioning of trace elements in digestive gland, kidney and gills of native *Octopus vulgaris* (Portugal). Marine Environmental Research.

Abstract

Concentrations of V, Co, Cu, Zn, As, Cd and Pb and their sub-cellular distributions were analyzed in digestive gland, kidney and gills of the common octopus, *Octopus vulgaris* caught from three areas of the Portuguese coast characterised by contrasting metal contamination. The observed element partitioning by tissues may be separated in four patterns: digestive gland>kidney>gills for Co and Cd; digestive gland>kidney, gills for V and Zn; similar levels of Cu in the three analysed tissues; kidney, gills> digestive gland for As. The elements present in digestive gland, kidney and gills are largely stored in the cytosolic fraction (HSP), in particular V, Co and As. The partitioning of Pb was mainly between HSP and granules. Good linear log-log relationships between levels in the organelles and cytosolic fraction and in the organelles and the whole digestive gland, kidney and gills were obtained for Cd and Co. The role of the elements in the cells, and consequently their association with the sub-cellular fraction, seems to superimpose the response as a function of availability in the tissue.

Introduction

Trace elements are unequally distributed among tissues of marine organisms. The function of the tissue, namely in the depuration or elimination of elements in concentrations exceeding the requirement needs, has been invoked to explain the elevated levels of essential and non-essential elements (Rainbow, 2002; Bustamante et al., 2002; Vijver et al., 2004). However, element partitioning among tissues is highly dependent of biochemical processes occurring within cells (Langston et al., 1998). Following absorption into the circulatory system, elements are transported to specific organs. When an element is incorporated into a molecule (e.g. hemocyanin or metalloenzymes) it enters the routine metabolic pathways. Trace elements in excess amounts are complexed and removed from the interaction with other biologically important molecules, either by sequestration or excretion (Engel and Brouwer, 1984). The binding of an “inappropriate” metal to a metal-sensitive site, like organelles and enzymes, is often associated with detoxification mechanisms (Simkiss and Taylor, 1982; Phillips and Rainbow, 1989; Bustamante et al., 2002) and could be an indicator of metal-induced stress (Wallace et al., 2003; Campbell et al., 2005). Manifestations of sub-lethal toxicity can coincide with changes in sub-cellular partitioning, particularly in cases where there is saturation of certain metal detoxification systems (Sanders et al., 1983; Wallace et al., 2003). The involvement of the sub-cellular compartments in metal sequestration has been investigated in laboratory experiments with molluscs (e.g. Bebianno and Langston, 1991; Hylland et al., 1994; Roméo and Gnassi-Baelli, 1995). Less data is available for metal sub-cellular partitioning in cephalopods (Tanaka et al., 1983; Finger and Smith, 1987; Bustamante et al., 2002) and these works consider mostly the partition between soluble and insoluble fractions.

This work reports the levels of V, Co, Cu, Zn, As, Cd and Pb in granules, mitochondria, lysosomes plus microsomes, heat-denaturable proteins and heat-stable proteins of digestive gland, appendage renal (herein designated kidney), and gills of *Octopus vulgaris* captured from three areas of the Portuguese coast. The areas were selected taking into account the different metal concentrations in tissues of *O.*

vulgaris along the Portuguese coast (Raimundo et al., 2004, 2005; Napoleão et al., 2005; Seixas et al., 2005a,b), presumably reflecting different metal availability in water and seston (Caetano and Vale, 2003). The three selected tissues are recognised as compartments of storage (digestive gland), elimination (kidney) and uptake (gills) of contaminants (Miramand and Bentley, 1992; Rainbow and Phillips, 1993; Bustamante et al., 2002; Raimundo et al., 2004). The work tests the hypothesis of sub-cellular partitioning of trace elements at different concentrations varying among digestive gland, kidney and gills.

Materials and methods

Samples

Eighteen common octopuses, *Octopus vulgaris*, were collected from commercial catches landed in Matosinhos (n=6), Olhão (n=6) and Cascais (n=6), situated in the NW, W and SE coast of Portugal, respectively (Figure 3.2.1). Octopuses were captured in November 2007 (Matosinhos and Olhão) and in February 2008 (Cascais). Each collected individual was weighted and mantle length and gender determined. The specimens were immediately dissected, digestive gland (without rupture of the outer membrane), kidney and gills of each organism being totally removed.

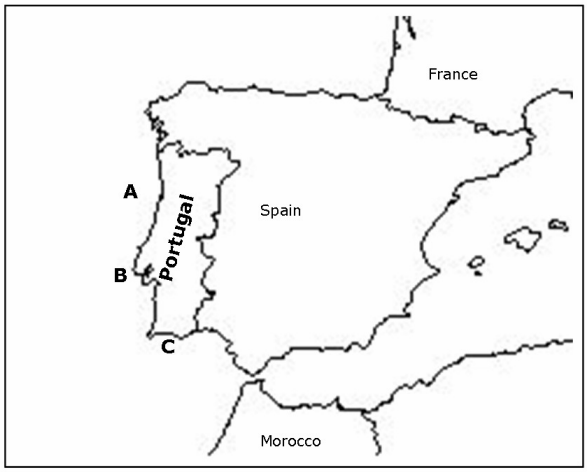

Figure 3.2.1 – Location of the three areas of capture of *Octopus vulgaris* in the Portuguese Coast: Matosinhos (A), Cascais (B) and Olhão (C).

Analytical methodology

Sub-cellular fractionation. A sub-sample of each fresh dissected tissue was homogenised at a dilution of 1:3 (wet mass:volume of buffer) in an ice bucket. The buffer consisted of Tris–HCl (10 mM, pH 7.4, and 0.15 M NaCl) and 1 mM PMSF (phenylmethylsulfonylfluoride, as protease inhibitor). The homogenation was performed by hand and completed in approximately 5 min. to minimize organelle breakage. Each homogenate was transferred to centrifuge tubes and subjected to differential fractionation for sub-cellular analyses. The procedure adapted from Wallace et al. (2003) and Campbell et al. (2005) comprised five operationally defined fractions. The three “particulate” fractions are granules, mitochondrias, and

lysosomes plus microsomes. The two "cytosolic" fractions consist of heat-stable proteins (HSP), including metallothioneins and glutathione, and heat-denaturable proteins (HDP) containing enzymes and other non-enzymatic proteins. The five fractions were obtained by the following centrifugation procedure: the aliquot was firstly centrifuged at 800g for 15 min at 4ºC (P1 and S1). The P1 that contained nuclei, unbroken cells, cell membranes and granules was re-suspended in initial buffer (1:3, m:v), heated at 100ºC for 2 min, 1N NaOH was added and heated again at 60-70ºC for 10min, after that a new centrifugation was made at 10 000g for 30 min at 20ºC. Two fractions were obtained, only the pellet (P2) with granules was further used. The supernatant S1, was centrifuged sequentially to separate P3 the mitochondria fraction, at 10 000g for 30 min at 4ºC, the lysosome and microsomal fractions (P4) were obtained by further centrifuging the supernatant at 100 000g, for 60 min at 4ºC. The "cytosolic" fractions (P5 and S5) were separated by heating the S4 at 80ºC for 10 min and centrifuging at 50 000g for 15 min at 4ºC. The heat-stable proteins (HSP) remain in the final supernatant. The five fractions obtained by the centrifugation procedure were lyophilized for trace element determination.

Trace elements. Trace elements were determined in lyophilised, grinded and homogenised samples of whole dissected tissues and pellets. Samples were 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. (1999). All lab ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Three procedural blanks were prepared using the same analytical procedure and reagents, and included within each batch of samples. Concentrations of Zn, Cu and Cd in the case of whole digestive gland were determined by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100) and V, Co, Zn, Cu, As, Cd and Pb by a quadropole ICP-MS (Thermo Elemental, X-Series). The accuracy of these analytical methods was assessed by the analysis of international certificate standards (DORM-1, DORM-2 – dogfish muscle; DOLT-1 – Fish liver and TORT-1, TORT-2 – lobster hepatopancreas). The results obtained were in good agreement with the certified values ($p<0.05$). Procedural blanks always accounted for less than 1% of the total trace element in the samples. All the results are given as medians and ranges in micro gram per gram of dry mass tissue ($\mu g\ g^{-1}$; dm).

Statistical analyses

Prior to statistical analyses, metal concentrations were tested for normality and equality of variances. Non-compliance with parametric ANOVA assumptions led to employment of the Kruskal-Wallis H (KW-H) and Mann-Whitney (U) non-parametric tests. Statistical tests were used to evaluate the existing differences between metal concentrations in the digestive gland, kidney and gills and pellets, and to compare the biological parameters of individuals from the three sampling areas. The significance for statistical analyses used was always $\alpha = 0.05$. Statistical analyses were performed using the STATISTICA 6.0 (Statsoft). Principal component analysis (PCA) was used to describe the variability of the results (Arfi et al., 1983). In this statistical method, each sample corresponds to a point multi-dimensional space and the

distance between points is proportional to the chemical composition dissimilarities of the corresponding samples.

Results

Biological data

Table 3.2.1 gives the size, weight and the proportion female:male of the sampled octopuses. Both size and mass of the individuals varied over similar ranges and were not significantly different in specimens from the three areas of capture ($p>0.05$, KW-H). Gender was also similar in individuals from these areas. Differences of these variables between the two sampling periods have also no statistical validity ($p>0.05$, KW-H).

Table 3.2.1 - Size (mm), weight (g) and female:male proportion of *Octopus vulgaris* captured in the three sampling areas along the Portuguese coast.

Areas	Size range (mm)	Weight range (g)	Female:male
Matosinhos	146-165	1162-1399	3:3
Cascais	135-160	1120-1570	2:4
Olhão	165-205	1231-1957	3:3

Influence of tissue and sampling area on trace element variability

The absence of relationships between metal concentrations and the abovementioned biological parameters allows treating data from each sampling area independently of the size/weight and gender of the individuals. Because concentrations of V, Co, Cu, Zn, As, Cd and Pb varied within broad ranges, a principal component analysis was applied in order to identify which factor, analysed tissue or sampling area, better explained the variability of element concentrations (Fig. 3.2.2). Factor I explains 55% of the variability and separates points representing digestive gland and gills/kidney. All determined elements were better associated with digestive gland, but As which was projected in a different quadrant and being preferentially associated with kidney and gills. Factor II explains only 18% of the variance and divides the sampling areas. The better geographic separation was obtained for digestive gland.

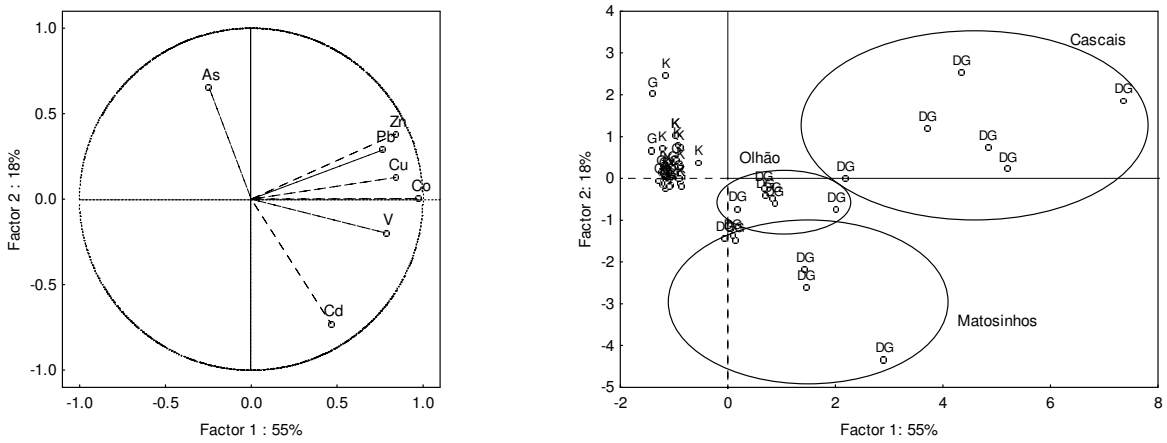

Figure 3.2.2 – Principal component analysis of metals in tissues of common octopus, *O. vulgaris* from the three capture areas. DG - digestive gland, K - kidney and G – gills.

Trace element concentrations in tissues

Since tissue presented a higher contribution to the observed variability in the element concentrations than sampling area, element partitioning among digestive gland, kidney and gills were searched combining data from the three areas. Figure 3.2.3 shows the median, the percentile 25% and 75%, minimum and maximum of V, Co, Cu, Zn, As, Cd and Pb concentrations in digestive gland, kidney and gills. Median concentrations (μg g^{-1}, dm) varied within broad intervals, being minimum values registered in gills and maximum in digestive gland: 0.75-4.2 (V); 0.10-10 (Co); 99-1374 (Zn); 187-481 (Cu); 0.10-78 (Cd); 0.37-6.3 (Pb). Conversely, As concentrations were low in digestive gland (median=24 μg g^{-1}) and high in kidney (median=69 μg g^{-1}). The observed element partitioning in tissues may be separated in four patterns: digestive gland>kidney>gills for Co and Cd ($p<0.05$, U); digestive gland>kidney, gills for V and Zn ($p<0.05$, U); similar levels of Cu in the three analysed tissues; kidney, gills> digestive gland for As ($p<0.05$, U).

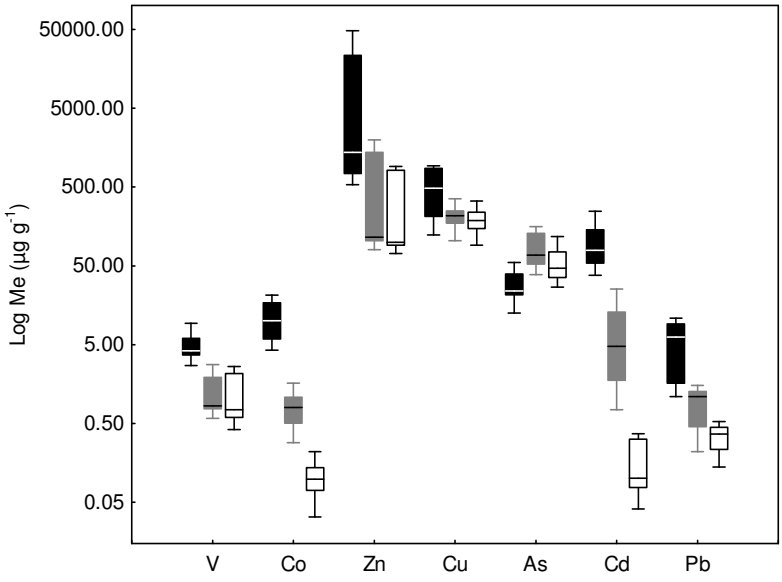

Figure 3.2.3 - Median, 25 and 75% percentile, minimum and maximum, of Log V, Co, Zn, Cu, As, Cd and Pb concentrations (µg g^{-1}, dry weight) in the digestive gland (black boxes), kidney (grey boxes) and gills (white boxes) of common octopus, *O. vulgaris* from the three areas of capture.

Trace element concentrations in sub-cellular fractions

Figure 3.2.4 shows the median, the percentile 25% and 75%, minimum and maximum of trace element content in granule, mitochondria, lysosome+microsome, HDP and HSP fractions of digestive gland, kidney and gills of octopus from the three areas. In general, metal concentration in each sub-cellular fraction ranged from a minimum in gills to a maximum value in digestive gland. Some elements showed a concentration interval of several orders of magnitude. For example, in the granule fraction the interval of Cu and Cd median concentrations were 67-1689 µg g^{-1} and 0.087-111 µg g^{-1}, respectively. Conversely, As showed low concentrations in all analysed fractions of digestive gland. Trace element concentrations in each separated fraction were statistically (U, $p<0.05$) compared among the three tissues. The following patterns were observed for each sub-cellular fraction:

Granule fraction: digestive gland>kidney>gills for Co, Cd and Pb; digestive gland>kidney, gills for V, Zn and Cu; kidney>digestive gland for As.

Mitochondria fraction: digestive gland>kidney/gills for V, Co, Zn, Cu, Cd and Pb; kidney>digestive gland for As.

Lysosome and Microsome fraction: digestive gland>kidney>gills for Co, Zn, Cd and Pb; kidney>digestive gland for As.

Heat-denaturable proteins fraction: digestive gland>kidney>gills for Co, Zn, Cu, Cd and Pb; kidney>digestive gland for As.

Heat-stable proteins fraction: digestive gland>kidney>gills for Co and Pb; digestive gland>kidney/gills for V, Zn, Cu and Cd; kidney/gills>digestive gland for As.

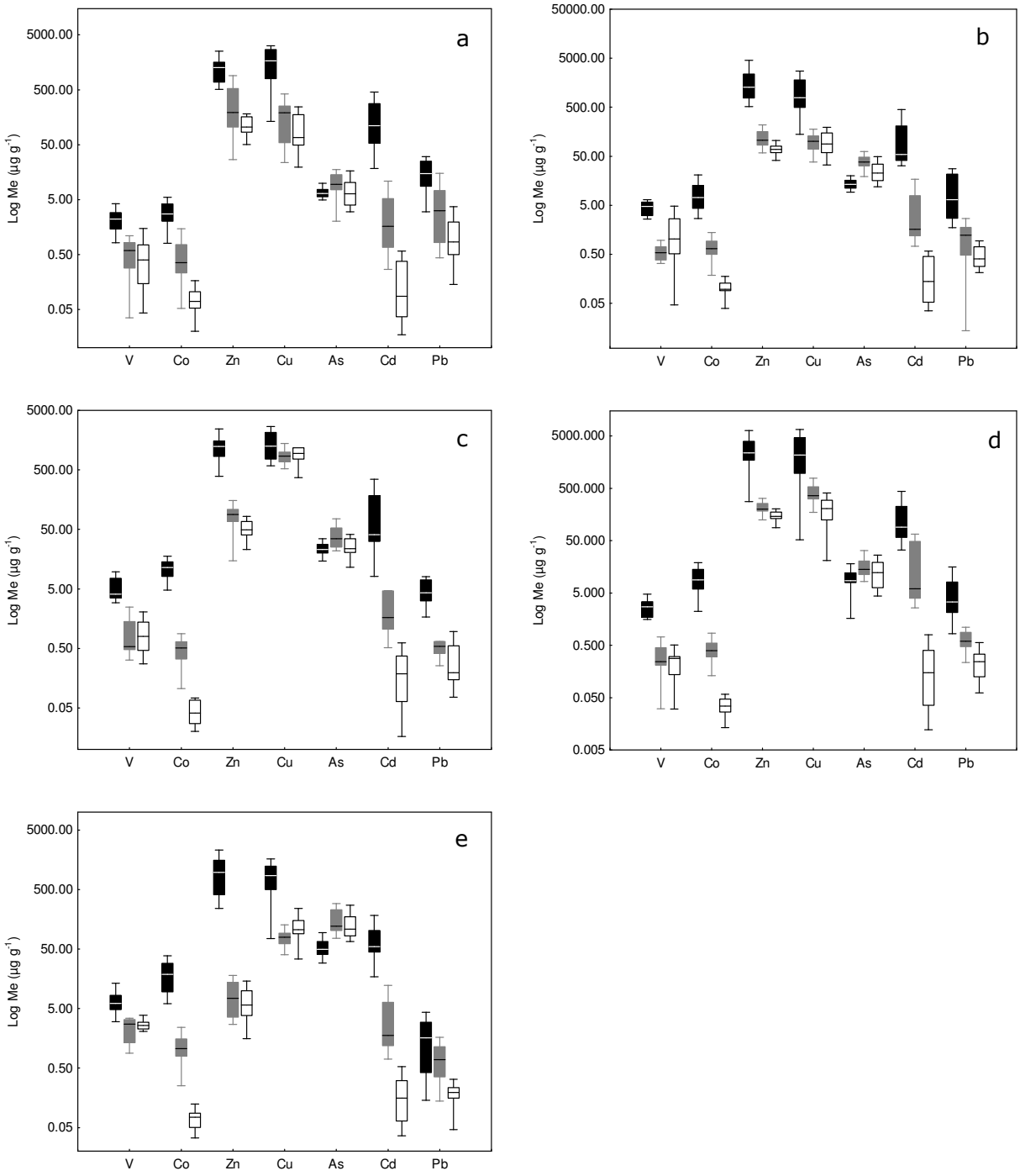

Figure 3.2.4 – Median, 25 and 75% percentile, minimum and maximum, of Log V, Co, Zn, Cu, As, Cd and Pb concentrations (µg g^{-1}, dry weight) in the digestive gland (black boxes), kidney (grey boxes) and gills (white boxes) of common octopus, *O. vulgaris* from the three areas of capture. a – Granules; b – Mitochondria; c – Lysosomes plus microsomes; d – HDP; e – HSP.

Trace-element content in sub-cellular fractions

Among the operationally separated sub-cellular fractions granules, mitochondria, lysosomes plus microsomes, heat-denaturable proteins (HDP) and heat-stable proteins (HSP), the last one constituted the heavier component in the three analysed tissues. In fact, the HSP accounted to 87-99% of the total tissue

mass, in contrast with the proportions of granules, mitochondria and HDP (<5.6%) and of lysosomes plus microsomes (0.064-1.2%). Differences between tissues were not significant (p>0.05, KW-H).

Tables 2 (end of the manuscript) gives the median, minimum and maximum proportions of V, Co, Zn, Cu, As, Cd and Pb found at each sub-cellular fraction of digestive gland, kidney and gills of octopus from the three sampling areas. The most noticeable aspect is that HSP contained 96-99% of V, 94-97% of Co and 98-99% of As. Negligible quantities of these elements were found in the other separated fractions. Cadmium, Cu and Zn were mainly stored in HSP (87-95%, 79-90% and 35-88%, respectively) and the remaining amounts distributed by HDP, granules and mitochondria, namely of kidney and gills. Quantities in the sub-cellular fractions of digestive gland were negligible. The partitioning of Pb was mainly between HSP (61-85%) and granules (5.7-25%).

Discussion

The examination of metal compartmentalization among sub-cellular fractions is highly important to interpret ecotoxicological consequences of metal partitioning and to provide a better understanding of potential mechanisms of toxicity and tolerance (Wallace et al., 2003). The methodology used in this work followed the current procedure to separate organelles, HDP and HSP fractions that are operationally defined by differential centrifugation. Consequently, the obtained results may contain potential artefacts, such as, breakage or clumping of particles, leakage of soluble constituents from organelles and overlap among sub-cellular fractions as reported in Wallace et al. (2003). Interpretation of the results is therefore within this procedural limitation.

Metal content in the cytosolic fraction

The largest quantities of the elements present in digestive gland, kidney and gills are stored in the cytosolic fraction (HSP). In particular the proportion of V, Co and As varied on average 96-99%, 94-97% and 98-99%, respectively. These values reflect the elevated contribution of HSP (94-97%) to the total mass in the sub-cellular fractions. However, other elements presented a smaller contribution of HSP: for Pb, 61% in digestive gland against 81 and 85% in kidney and gills; for Zn, 35 and 36% in kidney and gills, respectively, against 88% in digestive gland. The literature pointed to broader contribution of the soluble fraction in digestive gland. For example, Finger and Smith (1987) reported 78, 70 and 47% of Cu, Cd and Zn, respectively in the soluble fraction of digestive gland of the squid *Nototodarus gouldi*; Bustamante et al. (2002, 2006) showed that 42-86% of Cd, 38±8% of Pb, 40±9% of Zn and 64±9% of Co were present in the soluble fraction of various cephalopods; Tanaka et al., (1983) reported only 26% of Cd in the squid *T. pacificus*. To our knowledge no data has been published on As and V distribution in sub-cellular fractions of digestive gland of cephalopods. The elevated proportions of As (96-98%) and V (98-99%) in the cytosolic fraction of octopus tissues differed considerable from the nearly equal distribution of As between soluble and insoluble fractions found in the fur seals, green turtles and seabirds, and approximately 60% in the soluble fraction in the ringed seals and hawksbill turtles (Fujihara et al., 2003).

For V, low cytosolic retention (19%) was reported by Kunito et al. (2004) for Franciscana dolphin liver (*Pontoporia blainvillei*).

The elevated content observed in the HSP fraction may result from the presence of proteins with high affinity to metals such as metallothioneins (MT). A recent study on MT in octopus tissues (Raimundo et al., in press) pointed to the relation between these proteins and Cd in the digestive gland. Bustamante et al. (2006) also found a relation between Cu and MT in digestive gland of *S. officinalis*. On the other hand, the enhanced percentage of the analysed elements in this detoxifying fraction may be seen as a protection to the more sensitive fractions, organelles and HDP.

Metals in organelle fractions

As result of high affinity of V, Co and As to the cytosolic fraction, these elements are stored in small quantities in the insoluble fraction of the three analysed tissues (Table 3.2.2). A less extent storage was observed for Cu and Cd. In digestive gland of the *S. officinalis* the percentage of Zn, Cu, Cd, Co and Pb ranged from 11±4% (Cd) to 23±6 (Pb) (Bustamante et al., 2006). Although with little association with some elements, organelles are recognised as sensitive compartments to metal contamination and subsequent toxicity. The enhanced quantities in the granule fraction of kidney and gills for Zn and in digestive gland, kidney and gills for Pb may be indicative of existing detoxification mechanisms. Studies with isopods (Brown, 1978) showed that internal storage and detoxification of metals by metal-rich granules was related to increased tolerance of the individuals to environmental contamination. Wallace et al. (2003) included the metal-rich granules (MRG) in the biologically detoxified metal (BDM) group. However, granules are fairly ubiquitous in molluscs serving different functions, other than detoxification, within different cells in relation to the distribution of metals (Langston et al., 1998). The retention in the Lys+Mic fraction of Cu in kidney and gills is difficult to interpret since the two involved organelles have different roles. Lysosomes are known to store metals and cellular wastes products which cannot be degradated (Dallinger, 1993), but also to remove metals for eventual elimination (Giguère et al., 2006). The sequestration of elements can eventually result in adverse effects on lysosomal functions when detoxification capacity of the lysosomal system is overwhelmed (Sokolova et al., 2005). The destabilization of lysosomal membranes may result in the release of metals to the cytosol, potentially augmenting the toxicity. On the other hand, microsomes are associated with toxicity, due to the presence of fragments of the endoplasmic reticulum, were protein synthesis and transport occur and glycogen is stored (Jarosch et al., 2002; Bonneris et al., 2005).

Relationships of trace element concentrations between sub-cellular fractions and whole tissue

The key question approaching in this work is whether metal partitioning among sub-cellular fractions varies with the nature and function of digestive gland, kidney and gills in the octopus. Metal concentrations at each of the five sub-cellular fractions were plotted against the metal concentration in the whole tissue. Good linear log-log relationships between levels in the organelles and cytosolic fraction

and in the whole digestive gland, kidney and gills were obtained for Cd (R^2 between 0.81 and 0.96) and Co (R^2 between 0.60 and 0.91) (Figure 3.2.5). Although As and Pb exhibited the same tendency in the sub-cellular fractions (except for granules), only HDP-total (Pb) and HSP-total (As) relationships showed good correlations (R^2=0.66 for Pb and R^2=0.77 for As). Despite the different accumulation of Co, Cd, As and Pb in the digestive gland, kidney and gills, the concentration in each sub-cellular fraction varied proportionally with the concentration of the whole tissue. This variation appears to be similar in digestive gland, kidney and gills, regardless of their different nature and function in the octopus. These results can be seen as those fractions responding to element availability in the cell independently of the tissue. The relationships found for Co may be related to the fact that this element is an integral component of vitamin B12, which is important in cell division (Nolan et al., 1992). Moreover, studies in fish gill have demonstrated that Co ions compete with Ca^{2+} ions at the gill-water interface (Comhaire et al., 1994; Richards and Playle, 1998). Cadmium is a class B element, forming a wide range of covalent compounds and is therefore less likely to exist as free ions in solution (Simkiss and Mason, 1984). In addition, Cd may occasionally substitutes essential elements, like Zn and Cu in biological sub-cellular systems (Zauke and Petri, 1993), augmenting the number of potential ligands. The interference of As with the organelles has already been reported in the soft tissues of oysters (Ettajani et al., 1996). According to this work, an indirect evidence of an intracellular transport of As is possible due to the existence of organelle abnormalities, such as in the mitochondria and nuclei. A review by Marigomez et al. (2002) indicates that Pb is present in numerous cell types in molluscan tissues, being distributed both in lysosomes and cytosol. The good HDP-total correlation observed for Pb is probably related to the preferential association of this element with high molecular mass ligands (e.g. macromolecules) like it was observed by Bustamante et al. (2006) in digestive gland of cuttlefish, *Sepia officinalis*. The same trend was already observed for octopus collected in the Portuguese coast (Raimundo et al., 2010).

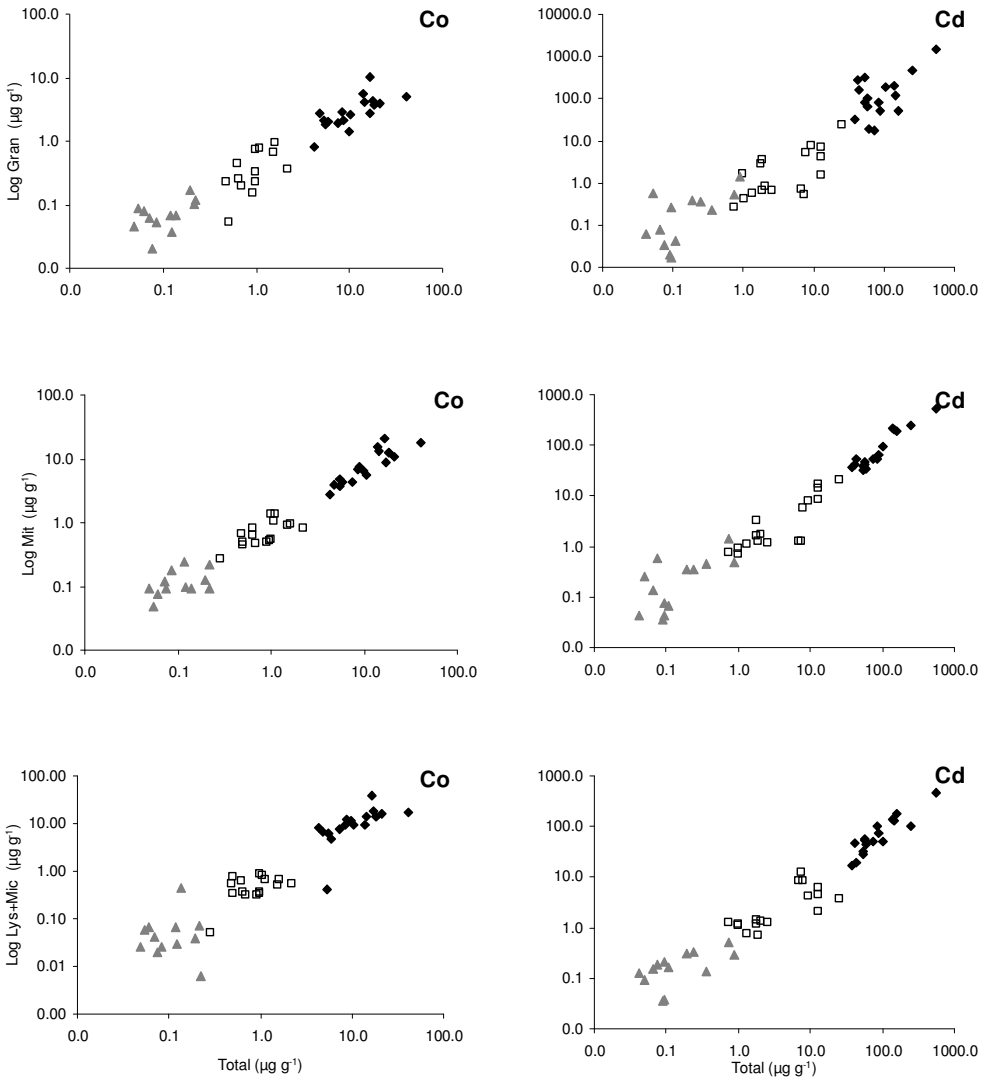

Figure 3.2.5 - Relationships between Log levels of Co and Cd (µg g^{-1}, dry weight) in: granules (Gran), mitochondria (Mit), lysosomes plus micrososmes (Lys+Mic), HDP and HSP and the whole digestive gland (♦), kidney (□) and gills (▲) of *O. vulgaris*.

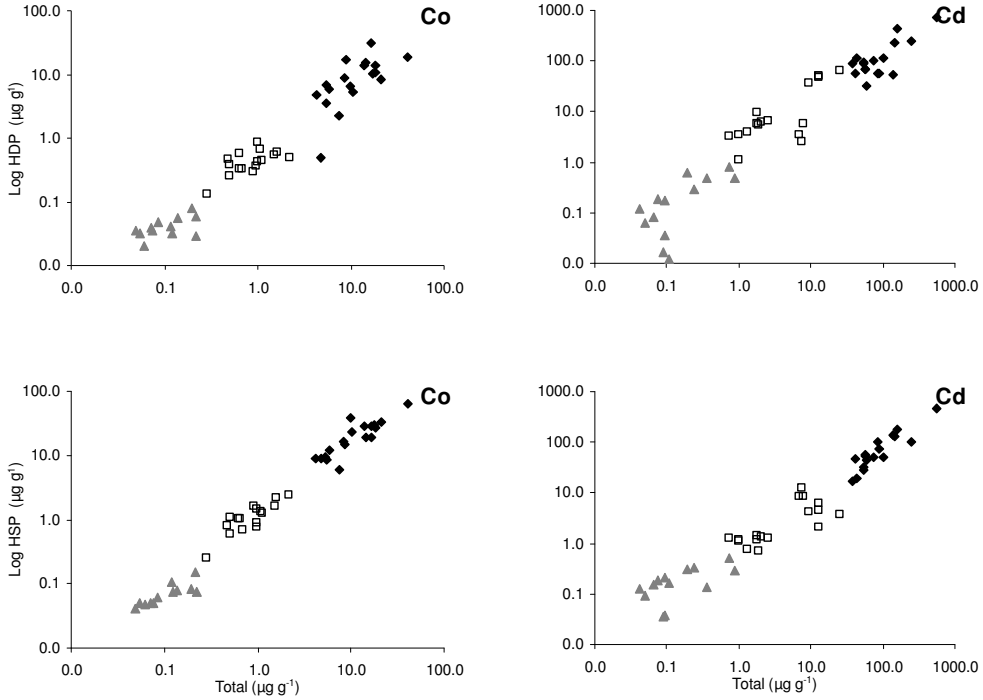

Figure 3.2.5 (Continued) – Relationships between Log levels of Co and Cd (µg g^{-1}, dry weight) in: granules (Gran), mitochondria (Mit), lysosomes plus micrososmes (Lys+Mic), HDP and HSP and the whole digestive gland (♦), kidney (□) and gills (▲) of *O. vulgaris*.

Poor relationships between fractions and whole tissue were obtained for V, Cu and Zn. The lack of proportionality, like obtained for the other elements, suggests a broader affinity of these elements for various types of ligands in the cells. A broad range of proteins might act as metal chaperone for delivering V to target proteins in some tissues or cells, being involved in the accumulation and reduction of this element (Yoshihara et al., 2008). Inside the cell V is reduced to cationic V (III) and V (IV) ions which are trapped inside (Kustin et al., 1983). For essential elements, Cu and Zn, the patterns of storage are more or less similar since metallothionein (HSP) and insoluble ligands are concurrently involved in detoxified storage (Amiard et al., 2008). Copper and Zn are involved in numerous metabolic functions and regulation mechanisms (Langston et al., 1998). The role of the elements in the cells, and consequently their association with the sub-cellular fraction, seems to superimpose the response existing as a function of availability in the whole tissue.

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Table 3.2.2 – Sub-cellular mass (%), V, Co, Zn, Cu, As, Cd and Pb levels (µg g^{-1}, dry weight) and sub-cellular distribution in the digestive gland, kidney and gills of *O. vulgaris*.

		Fractions mass (%)	V	Co	Zn	Cu	As	Cd	Pb
			% of cellular fraction to element content in whole tissue						
Granules	Dig. Gland	1.2 (0.19-5.0)	0.71 *(0.17-3.0)*	0.27 *(<0.1-1.4)*	2.3 *(1.2-5.8)*	1.9 *(0.38-4.0)*	0.24 *(<0.1-0.94)*	2.3 *(0.40-8.3)*	25 *(13-67)*
	Kidney	0.69 (0.35-1.7)	0.62 *(<0.1-5.5)*	0.84 *(0.16-4.8)*	12 *(5.9-30)*	1.3 *(0.43-9.7)*	0.13 *(<0.1-0.39)*	0.83 *(<0.1-4.1)*	11 *(1.1-35)*
	Gills	0.62 (<0.1-4.6)	0.39 (<0.1-1.6)	1.6 *(0.24-6.1)*	16 *(6.0-34)*	1.3 *(0.36-6.7)*	0.10 *(<0.1-0.56)*	1.2 *(0.23-7.8)*	5.7 *(2.1-18)*
Mitochondria	Dig. Gland	1.2 (0.18-4.6)	1.1 *(0.27-4.2)*	0.55 *(0.12-2.6)*	1.4 *(0.18-8.3)*	1.6 *(<0.1-6.3)*	0.43 *(<0.1-2.8)*	1.8 *(0.20-8.5)*	3.2 *(0.41-14)*
	Kidney	0.84 (0.12-2.1)	0.24 *(<0.1-0.43)*	0.82 *(0.14-4.3)*	11 *(3.2-41)*	1.6 *(0.27-5.4)*	0.46 *(<0.1-6.4)*	0.91 *(<0.1-4.8)*	1.2 *(<0.1-4.2)*
	Gills	0.42 (<0.1-1.5)	0.70 *(0.12-3.7)*	2.7 *(0.49-8.2)*	9.5 *(1.9-16)*	1.7 *(0.18-5.8)*	0.30 *(<0.1-3.0)*	1.4 *(0.10-3.5)*	2.1 *(0.18-9.0)*
Lysosomes+ Microsomes	Dig. Gland	0.41 (0.10-1.1)	0.35 *(<0.1-3.0)*	0.25 *(0.16-0.90)*	0.62 *(0.19-2.3)*	0.61 *(<0.1-4.2)*	0.23 *(<0.1-1.9)*	0.52 *(0.16-1.1)*	1.0 *(0.10-3.3)*
	Kidney	0.44 (0.13-1.2)	0.20 *(<0.1-0.69)*	0.25 *(<0.1-0.69)*	2.3 *(0.19-5.8)*	4.6 *(1.1-7.7)*	0.14 *(<0.1-0.40)*	0.49 *(<0.1-1.8)*	0.36 *(0.13-1.3)*
	Gills	0.20 (<0.1-0.83)	0.17 *(<0.1-0.56)*	0.32 *(<0.1-0.51)*	1.8 *(0.25-6.4)*	4.5 *(2.3-7.6)*	0.12 *(<0.1-0.79)*	0.44 *(0.15-1.5)*	0.35 *(0.13-2.3)*
HDP	Dig. Gland	1.8 (0.38-3.0)	0.75 *(0.32-4.4)*	0.90 *(0.32-2.6)*	5.0 *(1.5-8.9)*	2.2 *(0.96-7.5)*	0.40 *(<0.1-0.72)*	3.2 *(1.2-10)*	4.2 *(0.87-9.0)*
	Kidney	1.7 (<0.1-3.0)	0.40 *(0.12-1.0)*	1.0 *(0.26-2.3)*	26 *(10-69)*	7.7 *(6.2-19)*	0.24 (<0.1-1.1)	7.7 *(0.50-14)*	1.4 *(0.66-5.2)*
	Gills	1.0 (0.16-2.4)	0.28 *(<0.1-0.60)*	1.1 *(0.20-3.6)*	26 *(10-51)*	3.5 *(0.82-7.5)*	0.19 *(0.10-0.90)*	1.5 *(0.16-3.3)*	1.8 *(0.86-5.1)*
HSP	Dig. Gland	94 (87-99)	96 *(92-100)*	97 *(95-99)*	88 *(72-98)*	90 *(66-97)*	98 *(96-100)*	88 *(80-97)*	61 *(20-96)*
	Kidney	96 (95-98)	98 *(92-100)*	95 *(90-99)*	35 *(20-87)*	79 *(47-87)*	99 *(93-100)*	87 *(29-99)*	81 *(63-98)*
	Gills	97 (94-99)	99 *(96-100)*	94 *(77-97)*	36 *(23-79)*	87 *(63-94)*	99 *(96-100)*	95 *(81-98)*	85 *(53-94)*

Chapter 3.3

Association of Zn, Cu, Cd and Pb with protein fractions and sub-cellular partitioning in the digestive gland of *Octopus vulgaris* living in different metal exposure

Raimundo, J., Vale, C., Duarte, R., Moura, I. (2010). Association of Zn, Cu, Cd and Pb with protein fractions and sub-cellular partitioning in the digestive gland of *Octopus vulgaris* living in different metal exposure. Chemosphere. Doi:10.1016/j.chemosphere.2010.08.029

Abstract

The concentrations of Zn, Cu, Cd and Pb were determined in the protein fractions of digestive gland and in the whole digestive gland of *Octopus vulgaris* collected in two areas of the Portuguese coast. Approximately 95% of Zn, 99% of Cu, 85-96% of Cd and 77-86% of Pb were stored in the cytosol, suggesting the predominance of cytosolic proteins in trapping these elements. Gel filtration chromatography evidenced the presence of two major groups of proteins, with high molecular weight (HMW, 144 000-130 000 Da) and low molecular weight (LMW, 11 000-6 000 Da). The following metal-protein associations were found: Zn was distributed between HMW and LMW; Cu and Cd in LMW proteins with a minor association with HMW; and Pb in HMW proteins. The strong positive correlations between Cd, Zn and Cu and LMW proteins point to the presence of metalloproteins with high affinity to these elements, although the shift observed between the maximum of the ratio 254:280 nm and metal concentrations in the chromatographic profiles suggests that metallothioneins have not a full participation in binding these elements.

Introduction

Cephalopods are known for their ability to accumulate high levels of essential and non-essential elements in digestive gland (e.g. Martin and Flegal, 1975; Miramand and Guary, 1980; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante et al., 1998a, b; Bustamante et al., 2000). Such high retention of potentially toxic elements is predictably associated with detoxification mechanisms (Simkiss and Taylor, 1982; Phillips and Rainbow, 1989; Bustamante et al., 2002). A well documented detoxification strategy involves the association of metals to metallothioneins (MTs) (Roesijadi, 1992), which plays an important role in the absorption, metabolism, homeostasis and storage of both essential and non-essential elements (Stone and Overnell, 1985; Chang and Huang, 1996; Roesijadi, 1996). Although MTs are known to be present in marine invertebrates (Bebianno and Langston, 1991; Roesijadi, 1992; Viarengo and Nott, 1993), these proteins have been quantified in octopus (Raimundo et al., in press). However, proteins with low, intermediate or high molecular weight have been also reported as potential binding sites for trace metals (Tanaka et al., 1983; Finger and Smith, 1987; Castillo and Maita, 1991; Craig and Overnell, 2003).

Octopus vulgaris has a worldwide distribution, living of sedentary habits in coastal waters and susceptible of being influenced by local environmental conditions (Mangold, 1983). High levels of metals have been registered in digestive gland of O*ctopus vulgaris* presumably due to higher metal availability in the environment or food (Renzoni et al., 1973; Miramand and Guary, 1980; Soldevilla, 1987; Raimundo et al., 2004, 2005), but to our knowledge the involvement of metal-binding proteins have not been documented.

This paper reports associations between metal and proteins fractions (by molecular weight) of digestive gland of *Octopus vulgaris*, and examines possible interactions between Cd, Zn and Cu, in low molecular weight proteins.

Material and Methods

Composite Samples

Octopuses, *Octopus vulgaris*, were collected in the Portuguese coast, Matosinhos (NW) and Olhão (SE), where previous works have shown contrasting metal accumulation in digestive gland (Raimundo et al., 2004, 2005) (Figure 3.3.1).

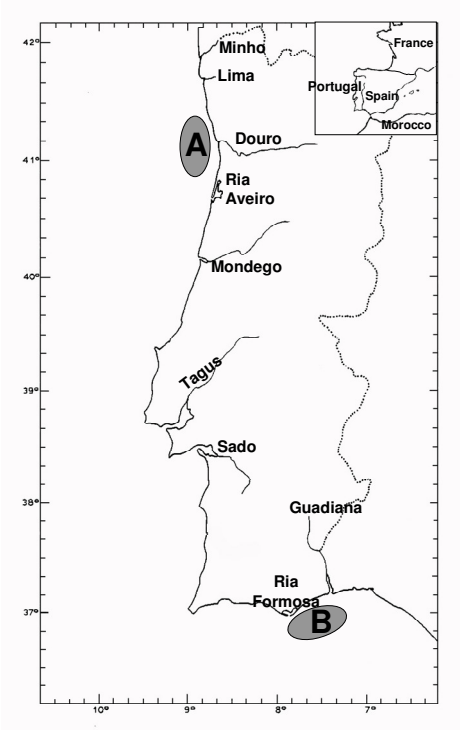

Figure 3.3.1 – Location of the two sampling areas of *O. vulgaris* in the Portuguese coast: Matosinhos and Olhão.

Each specimen was weighted, measured and digestive gland removed and frozen at -80ºC. Table 3.3.1 gives the number of sampled individuals, weight and mantle length. Prior to the preparation of composite samples Zn, Cu, Cd and Pb were determined in whole digestive gland of single individuals. The digestive gland of specimens from each sampling area presenting metal concentrations that varied within ±20% was considered for the preparation of composite samples. Composite samples of digestive gland were prepared with specimens from each area.

Table 3.3.1 - Number of individuals (n), and the ranges of weight (g) and mantle length (mm) of *Octopus vulgaris* captured in the Portuguese coast.

Sampling areas	n	Weight (g)	Length (mm)
Matosinhos	16	796 - 1433	125 – 170
Olhão	17	827 - 1520	135 – 165

Protein purification

One composite sample from each sampling area, which showed contrasting metal concentrations, were homogenised at a dilution of 1:3 (wet weight:volume of buffer) in an ice bucket. The buffer consisted of Tris-HCl (10 mM, pH 7.4, and 0.15M NaCl) and 1mM PMSF (phenylmethylsulfonylfluoride, as protease inhibitor). The homogenate was centrifuged at 100 000 x g for 1h at 4ºC and the supernatants were carefully pipetted off and immediately stored at -80ºC. The soluble clear solution was applied to a gel filtration column (Sephadex G-75, 2.6 x 89 cm) equilibrated with Tris-HCl (10mM, pH 7.4, 0.15M NaCl). The column temperature was maintained at 4ºC. The supernatants were applied to the column using volumes of 5mL. Elution was performed at a flow rate of 0.3 mL/min and fractions of approximately 4mL were collected. The column was calibrated with standards of the different molecular weight: blue dextran (approx. 2 000 000 Da), albumin (67 000 Da), ovalbumin (43 000 Da), chymotrypsinogen A (25 000 Da) and ribonuclease A (13 700 Da). Absorption at 254 and 280 nm as well as the concentrations of Zn, Cu, Cd and Pb were measured in each fraction.

Metal analyses

Zinc, Cu, Cd and Pb were analysed in lyophilised samples of individual digestive glands, composite samples of whole digestive glands and protein fractions. Samples, with the exception of protein fractions that were analysed directly, were digested with a mixture of HNO_3 (sp, 65% v/v) and H_2O_2 (sp, 30% v/v) according to the method described in Ferreira et al. (1990). All lab ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Metal concentrations were determined by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100) or graphite furnace atomic absorption spectrometry (Perkin Elmer, Zeeman 4110ZL). The accuracy of these analytical methods was assessed by the analysis of international certificate standards (TORT-1 and TORT-2). Measured and certified values did not differ significantly ($p<0.05$) (Table 3.3.2).

Table 3.3.2 - Zinc, Cu, Cd and Pb concentrations (nmol g^{-1}, dry wt) of lobster hepatopancreas (TORT-1 and TORT-2) (NRCC) determined in the present study and certified values.

Standard	Zn	Cu	Cd	Pb
	(nmol g^{-1})			
TORT-1				
Present study	2562±202	5964±378	219±26	54±13
Certified	2707±153	6908±346	234±19	50±10
TORT-2				
Present study	2640±174	1506±78	254±15	1.6±0.35
Certified	2753±92	1668±157	238±5.3	1.7±0.63

Statistical analysis

Prior to statistical analysis, metal concentrations and biological parameters were tested for normality and equality of variances. The Kruskal-Wallis test was applied to all data in order to detect differences between metal concentrations and sampling areas. The significance used for statistical analyses was $p<0.05$. The statistical analyses were performed using the STATISTICA 6.0 Statistical Software System.

Results and Discussion

Metal concentrations

Median, 25 and 75% percentile, minimum and maximum of Zn, Cu, Cd and Pb concentrations in digestive gland of *O. vulgaris* collected in Matosinhos and Olhão are presented in Figure 3.3.2. Zinc and Cu were the most abundant elements (medians of 21 and 20 μmol g^{-1}, respectively) reaching two to three orders of magnitude above the levels observed for Cd (median 0.39 μmol g^{-1}) and Pb (median 0.019 μmol g^{-1}). The enhancement of Zn and Cu levels is probably associated with the involvement of these elements in a number of metabolic functions, such as in metal-dependant enzymes (Craig and Overnell, 2003). Moreover, Cu is used in haemocyanin as a respiratory pigment (Bustamante et al., 2000). Zinc and Cu presented similar concentration ranges to those reported for the digestive gland of other cephalopods (Miramand and Guary, 1980; Soldevilla, 1987; Raimundo et al., 2004; Napoleão et al., 2005; Raimundo and Vale, 2008). No differences were obtained between sampling areas. Cadmium concentrations varied within a broad interval, from 0.087 to 2.2 μmol g^{-1}. Specimens captured in Matosinhos presented higher levels than in Olhão, being median concentrations 2.2 and 0.27 μmol g^{-1}, respectively. This difference is in line with previous work showing that Cd accumulation in digestive gland of octopus varied geographically along the Portuguese coast (Raimundo et al., 2004, 2005; Napoleão et al., 2005). The levels of Pb were above the values reported for *Sepia officinalis* and *Eledone cirrhosa* from English Channel (Miramand and Bentley, 1992). Concentrations in octopus collected in Olhão were slightly higher than in Matosinhos, which is in line with findings by Raimundo et al. (2004) and Napoleão et al. (2005) for the same species in the Portuguese coast.

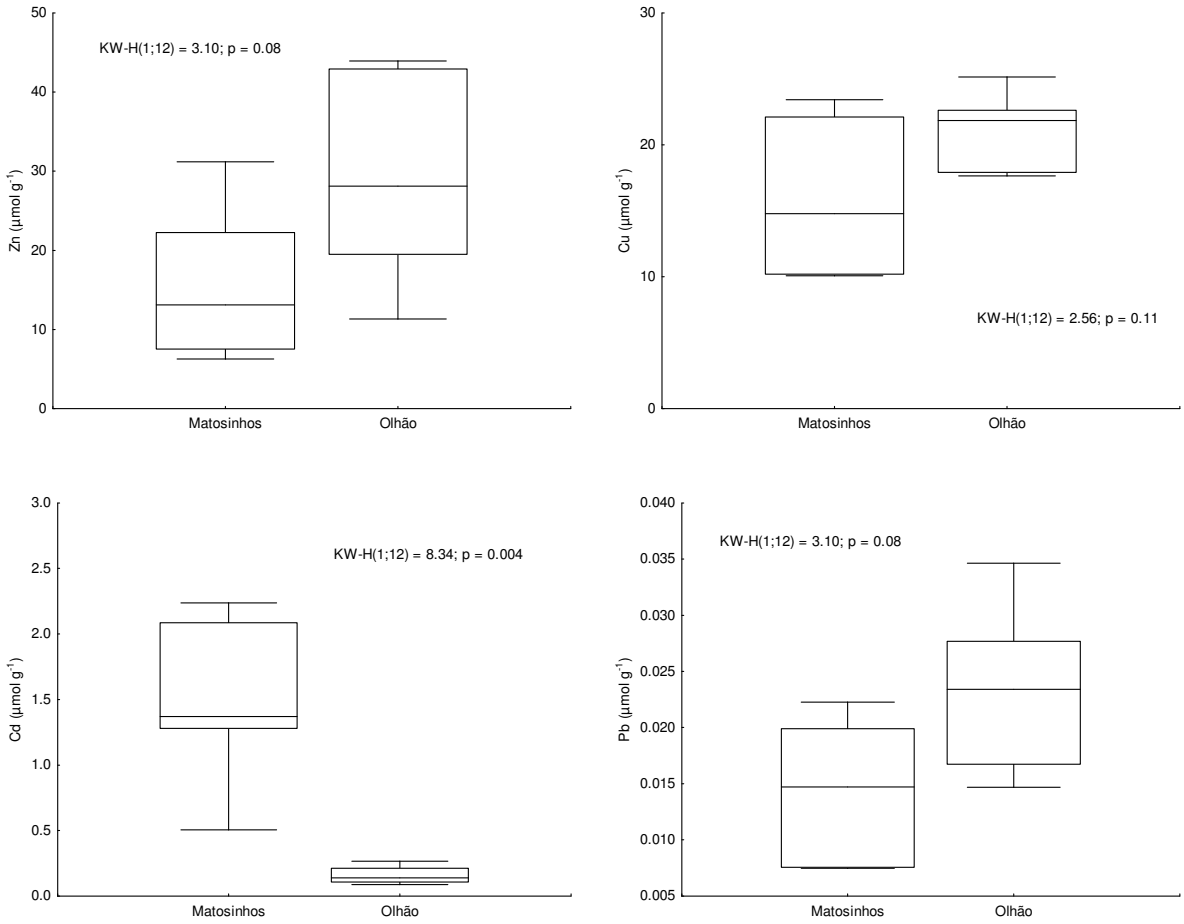

Figure 3.3.2 – Median, 25 and 75% percentile, minimum and maximum of Zn, Cu, Cd and Pb (µmol g^{-1}, dry weight) concentrations in composite samples of *Octopus vulgaris* digestive gland captured in Matosinhos and Olhão.

Cytosolic fraction

Approximately 95% of Zn, 99% of Cu, 85-96% of Cd and 77-86% of Pb were in the cytosolic fraction. These values indicate the dominant role of cytosolic proteins in trapping metals. High Cd contents have been reported in cytosol of the digestive gland from several cephalopod species (Finger and Smith, 1987; Tanaka et al., 1993; Bustamante et al., 2002; Bustamante and Miramand, 2005). Less data is available for Pb in cytosolic fraction of marine organisms. However, the proportion of Pb observed in this study is relatively high when compared to the 38±8% registered in *Sepia officinalis* (Bustamante et al., 2006), the 50% accumulated in cytosol of sea turtles liver (Anan et al., 2002), and the 33% in digestive gland of the scallop, *Chlamys varia* (Bustamante and Miramand, 2005). Also the proportions of Zn and Cu bound to cytosol are higher than the values reported by Craig and Overnell (2003), with only 35% of Cu and 43 % of Zn. The difference may be related to the high levels of metals present in digestive gland of the analysed octopus.

Chromatographic analysis

The chromatograms of digestive gland cytosol are presented in Figure 3.3.3. Profiles of the samples from Matosinhos and Olhão evidenced the presence of two major groups of proteins, with high molecular weight (HMW, 144 000-130 000 Da) and low molecular weight (LMW, 11 000-6 000 Da). Zinc was distributed between HMW and LMW, which is in agreement with the affinity observed in the liver of squid, *Nototodarus gouldi* (Finger and Smith, 1987) and in digestive gland of *S. officinalis* (Bustamante et al., 2006). Otherwise, most of the soluble Cu and Cd were present in LMW proteins, with a minor association with HMW. The affinity of Cd to the two groups of MW proteins was better registered in the most contaminated sample (Matosinhos). Finger and Smith (1987) also observed in the squid digestive gland that Cd proteins partition differs with concentrations, being higher levels associated with more than one group of MW proteins. Moreover, Tanaka et al. (1983) found higher proportion of cytosolic Cd associated with LMW and HMW fractions in the squid, *T. pacificus*. The results obtained with octopus are in agreement of those studies. It is suggested that as Cd is accumulated, which happen with the increasing availability in environment or food, the element is progressively bound to HMW proteins. However, a different pattern was obtained for *S. officinalis* with enhanced Cd levels associated with HMW proteins. In contrast to the other metals, the majority of Pb in octopus digestive gland was found in HMW, being in line with findings by Bustamante et al. (2006) for cuttlefish. Although association of Cd, Cu and Zn with high, intermediate and low molecular weight proteins in the liver/digestive gland of the squid species, *N. gouldi*, *T. pacificus*, *O. borealijaponica*, *O. bartrami* and *L. forbesi*, has been reported (Tanaka et al., 1983; Finger and Smith, 1987; Castillo and Maita, 1991; Craig and Overnell, 2003), to our knowledge this is the first work reporting metal-binding proteins in *Octopus vulgaris.*

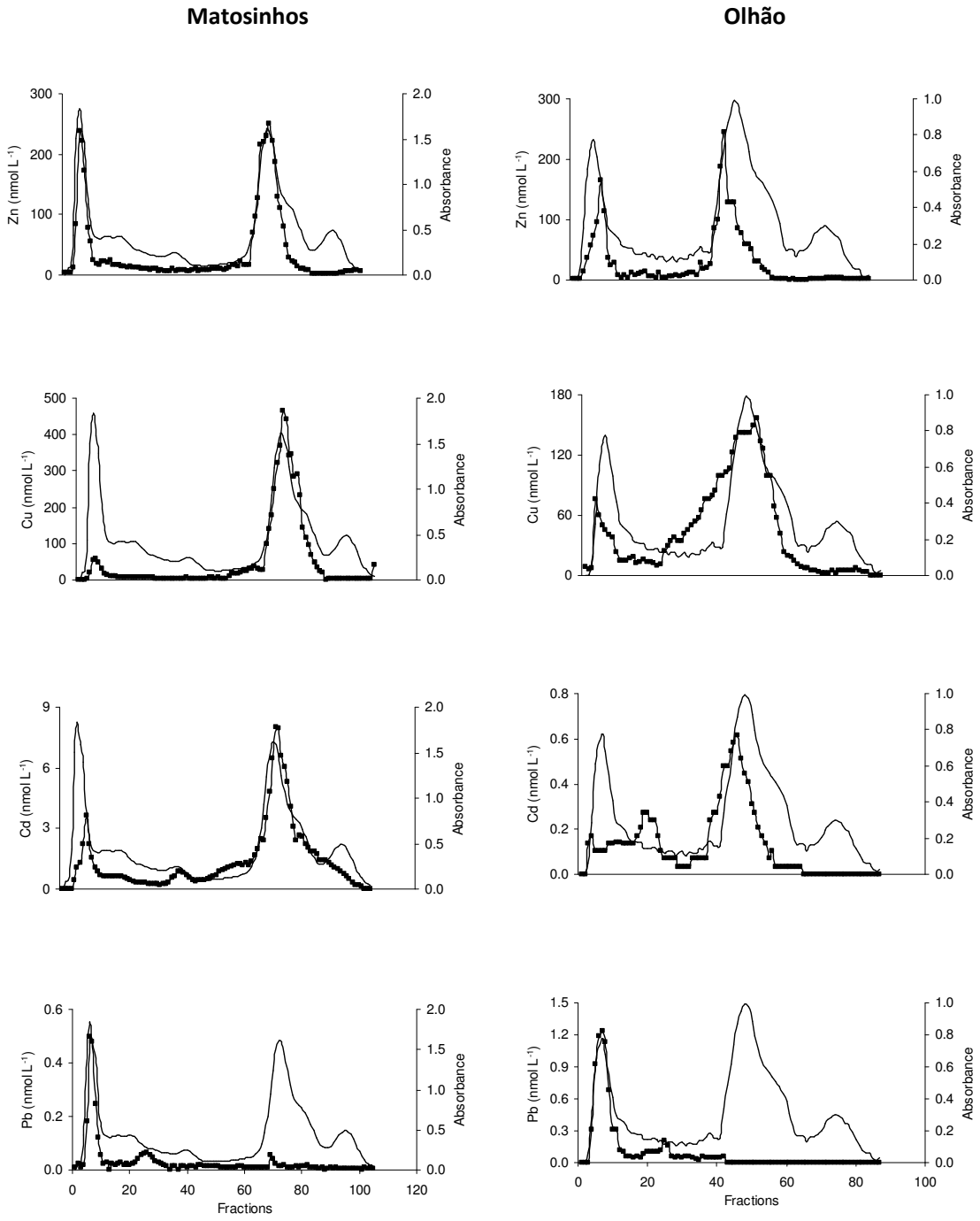

Figure 3.3.3 - Sephadex G-75 profiles of Zn, Cu, Cd and Pb concentrations (nmol L^{-1}) (■) in *Octopus vulgaris* digestive gland cytosol of samples from Matosinhos and Olhão and 280 nm absorbance (-).

Metal association with LMW proteins

The absorbance at 280 nm and 254 nm was measured at all fractions collected from the Sephadex G-75. Most of proteins absorb at 280 nm, due to the presence of aromatic amino acids, while metallothionein show higher absorption at 254 nm, due to absence or paucity of aromatic amino acids (Dallinger et al., 1989) and charge-transfer absorption of metal-thiolate bonds (Park et al., 2002; Craig and

Overnell, 2003). The ratio 254:280 nm has been invoked as a possible indicator of the presence of metallothioneins (Dallinger et al., 1989; Geret and Cosson, 2002; Park et al., 2002; Nam et al., 2005). Figure 3.3.4 compares the ratio 254:280 nm and Cd concentrations in the profile fractions of samples from Matosinhos and Olhão. The ratio 254:280 nm was below 1 at high molecular weight proteins, then increased at intermediate molecular weight proteins, and showed a pronounced peak at lower molecular weight proteins, indicating the paucity of aromatic amino acids. However, the maximum of the ratio 254:280 nm did not coincide with the maximum of Cd concentration in the profiles. This shift suggests that metallothioneins have not a full participation in the metal binding. These results are in agreement with Bustamante et al. (2002) and Craig and Overnell (2003). Nevertheless, a previous work with octopus containing twice levels of Cd in digestive gland pointed to MT-Cd as well as MT-Cr association (Raimundo et al., in press). A possible explanation for this discrepancy is the threshold value being achieved and as a response MT was induced to prevent Cd toxicity. Presumably, the lack of Cd-MT relationship observed in the present study means that Cd concentrations were below the threshold value. The same shift was observed for Zn and Cu concentrations.

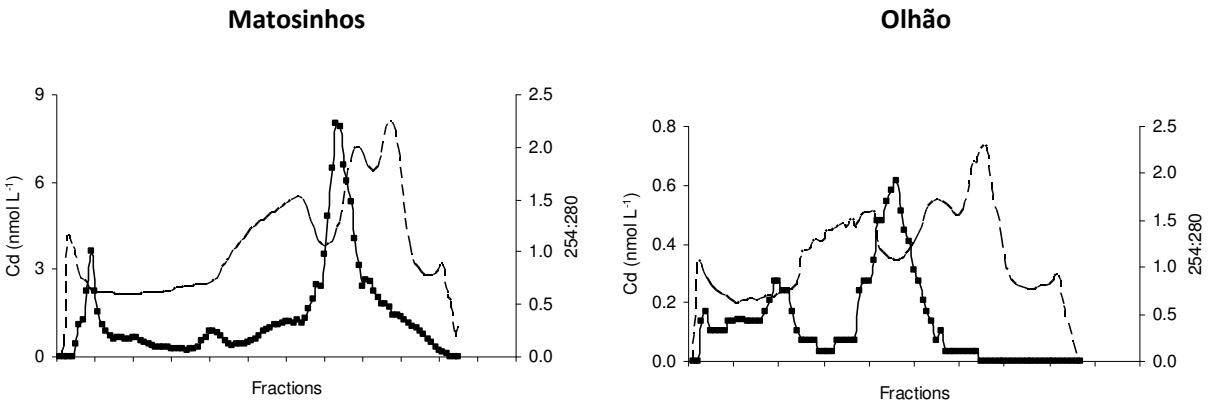

Figure 3.3.4 – Cadmium concentrations (nmol L^{-1}) (■) in the octopus digestive gland of samples from Matosinhos and Olhão and 254:280 nm ratio in the chromatographic profile (-).

Metal-metal relationships

Cadmium, Zn and Cu relationships were searched in the protein fractions and only at the peak corresponding to low molecular weight metals showed positive correlations (Figure 3.3.5). These strong correlations point that Zn, Cu and Cd have high affinity to metalloproteins that naturally bind these divalent ions. Cadmium is well correlated to both Zn and Cu in this protein fraction. However, as its concentration increased the molar Cd:Zn ratios changed from 3:1000 (sample from Olhão) to 23:1000 (sample from Matosinhos), and Cd:Cu ratio from 3:1000 to 14:1000. Interactions between Cd and Cu/Zn in biological structures of digestive gland of octopus is therefore expected, and can occur at absorption, distribution and excretion stages as suggested by Brzóska and Moniuszko-Jakoniuk (2001).

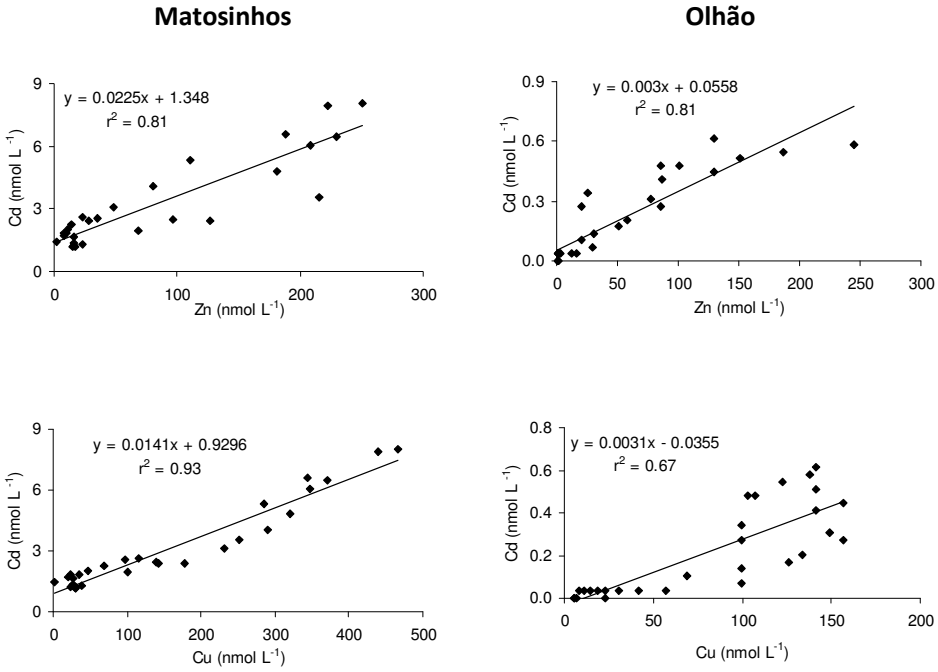

Figure 3.3.5 – Relations between Cd and Cu and Zn (nmol L^{-1}) in the peak with low molecular weight of the cytosol from the digestive gland of octopus from Matosinhos and Olhão.

In short, Pb exhibited an association to high molecular weight proteins while Zn, Cu and Cd showed link to high and low molecular weight proteins. Good relationships were observed between Cd and Zn/Cu in low molecular weight fractions. However, the poor relationship between metal concentrations and the ratio 254:280 nm suggest that levels were insufficiently low to trigger a detoxification mechanism involving metallothioneins.

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Chapter 3.4

Metallothioneins and trace elements in digestive gland, gills, kidney and gonads of *Octopus vulgaris*

Raimundo, J., Costa, P.M., Vale, C., Costa, M.H., Moura, I. (2010). Metallothioneins and trace elements in digestive gland, gills, kidney and gonads of *Octopus vulgaris*. Comparative Biochemistry and Physiology, Part C 152, 139-146.

Abstract

Metallothionein-like proteins (MT) and V, Cr, Co, Ni, Zn, Cu, As and Cd were determined in digestive gland, gills, kidney and gonads of *Octopus vulgaris*, from the Portuguese coast. To our knowledge these are the first data on MT in octopus. High concentrations (μg g^{-1}, dry mass) of Zn (48050) and Cd (555) were found in digestive gland, and MT reached levels one order of magnitude above the ones registered in wild bivalves. Significantly higher levels of MT in digestive gland and gills of specimens from A and B were in line with elevated Cd concentrations. Principal component analyses (PCA) point to MT-Cd and MT-Cr associations in digestive gland and gills. Despite the high levels of Zn in specimens from B, association with Zn was not obtained. Due to the affinity of MT to various elements, it should not be excluded the possibility of Cd replacing Zn in Zn-MT. Kidney presented higher levels of Cd, Co, Ni and As than gills and gonads, and in the case of As surpassing the levels in digestive gland, but PCA showed no relation with MT. Likewise the MT levels in gonads had no correspondence to the metal concentration variation.

Introduction

The effect of metals at sub-cellular and biochemical levels has been studied in several marine organisms (e.g. Bebianno and Serafim, 1998; Ng and Wang, 2004; Diniz et al., 2007). When accumulated metals in tissues reach the threshold level, detoxification mechanisms may be activated in order to prevent toxicity to the organisms. Sequestration and excretion are important pathways to eliminate metals from the interaction with biologically relevant molecules (Engel and Brouwer, 1984). A well-known detoxification strategy in marine invertebrates consists of inducing metallothioneins (MT), proteins to which several metals and metalloids have a high affinity (Bebianno and Langston, 1991; Roesijadi, 1992; Viarengo and Nott, 1993). These small cytosolic proteins (6-7 KDa, 57-75 amino acids) are characterised by high thiol content (18-20 cysteines per molecule), heat stability, and lack of aromatic amino acids (Viarengo, 1989; Viarengo and Nott, 1993; Simes et al., 2003; Vergani et al., 2007 and references herein). The high affinity of metals for MT provides a mechanism for protection against toxicity of non-essential elements (e.g., Cd), regulation of Zn and Cu at homeostatic levels (Roesijadi, 1992, 1996; Park et al., 2001), and protection of cells against oxidative stress by scavenging free radicals (Thornalley and Vasak, 1985). Various experimental works have proved that trace elements can act, at certain levels, as effective MT inducers (Bebianno et al., 1993; Bebianno and Serafim, 1998; Lueng and Furness, 2001; Ng and Wang, 2004; Shi and Wang, 2005). The production of MT has also been recorded in organisms exposed to complex mixtures of contaminants under environmental conditions (Geffard et al., 2002; Bebianno and Serafim, 2003; Smaoui-Damak et al., 2004).

Proteins with either low- or high-molecular mass have been reported as potential binding sites for trace metals in cephalopods (Finger and Smith, 1987; Castillo and Maita, 1991). Metallothioneins have been quantified in wild specimens, but relationships to metal concentrations have not been found (Craig and Overnell, 2003; Bustamante et al., 2002). *Octopus vulgaris* accumulates metals especially in digestive

gland (e.g., Bustamante et al., 1998b; 2000; Nessim and Riad, 2003; Raimundo et al., 2005; 2008) but, to our knowledge, specific research on MT has not yet been performed.

This paper reports the partitioning of V, Cr, Co, Ni, Zn, Cu, As and Cd and, for the first time, the levels of MT in digestive gland, gills, renal appendages (herein called kidney) and gonads of *O. vulgaris* captured in three areas of the Portuguese coast with different environmental contamination by metals and metalloids. Essential and non-essential elements were selected due to their different concentrations in tissues and eventually affinity to MT. The analysed tissues are recognised to mirror the input, elimination or storage of trace elements.

Material and Methods

Study areas

Metal concentrations in water and seston differ along the Portuguese coastal area, namely due to the exchanges with meso-tidal estuaries and coastal lagoon (Caetano and Vale, 2003). This work was carried out with octopuses captured from three coastal areas: Matosinhos-A, Cascais-B and Olhão-C (Figure 3.4.1). The area A is drained by large Iberian rivers, along which there are intensive industrial, agricultural and urban activities (Araújo et al., 2000). Enhanced levels of Cd and Cu in water column have been registered (Caetano and Vale, 2003). The area B has a strong influence of discharges from the Tagus estuary, including anthropogenic materials derived from Lisbon and nearby industries (Vale, 1990; Mil-Homens et al., 2009). The area C is influenced by small rivers crossing the Iberian Pyritic Belt with ores containing large quantities of Zn, Cu and Pb, minor Cd content and traces of Ni (Palanques et al. 1995; Elbaz-Poulichet and Leblanc, 1996).

Figure 3.4.1 – Location of the three areas of capture of *Octopus vulgaris* in the Portuguese Coast: Matosinhos (A, 41º 09.0' N08º 41.1' W), Cascais (B, 38º 36.0' N; 09º 27.2' W) and Olhão (C, 36º 55.0' N; 07º 52.7' W).

Sampling

Twenty three individuals of *Octopus vulgaris* (common octopus) were collected in November 2007 from catches of a trawling fishery vessel operating at areas A (n=6), B (n=11) and C (n=6). The sampled *O. vulgaris* were kept on ice until laboratory. Then each individual was weighted and mantle length and gender determined. The specimens were immediately dissected and digestive gland, gills, kidney and gonads of each octopus were totally removed without rupture of the outer membrane.

Metal determinations

Metals were determined in lyophilised, ground and homogenised samples after digestion 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 lab ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Three procedural blanks were prepared using the same analytical procedure and reagents, and included within each batch of samples. Concentrations of Zn, Cu and Cd (digestive gland) were determined by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100) and V, Cr, Co, Ni, As and Cd by a quadropole ICP-MS (Thermo Elemental, X-Series). The accuracy of these analytical methods was assessed by the analysis of international certificate standards (DORM-1, DORM-2 – dogfish muscle; DOLT-2 – Fish liver and TORT-1, TORT-2 – lobster hepatopancreas). The results obtained did not differ significantly ($p<0.05$) from the certified values. Procedural blanks always accounted for less than 1% of the total metal in the samples. The detection limits for the flame atomic absorption spectrometry analyses were 0.50 μg g^{-1} for Zn, 1.2 μg g^{-1} for Cu and 0.010 μg g^{-1} for Cd. For ICP-MS detection limits were: 0.0030 μg g^{-1} for V, 0.028 μg g^{-1} for Cr, 0.0020 μg g^{-1} for Co, 0.0080 μg g^{-1} for Ni, 0.23 μg g^{-1} for Zn, 0.020 μg g^{-1} for Cu, 0.74 μg g^{-1} for As and 0.0060 μg g^{-1} for Cd. All the results are given as medians and ranges in microgram per gram of tissue dry mass (μg g^{-1}, dm).

Quantification of metallothionein (MT)

Fresh samples of digestive gland, gills, kidney and gonads were homogenised in cold (4 ºC) TRIS–HCl 0.02M buffer (pH 8.6) using a Potter–Elvehjem homogenizer, in an approximate proportion of 1:3 tissue ww:buffer volume. Homogenates were centrifuged at 30 000 × g (1h at 4 ºC) and the supernatant (cytosol) was heated at 80 ºC for 10 min to denaturate non–heat resistant proteins. Heat–treated cytosols were then centrifuged at 50 000 × g (30 min h at 4 ºC) to precipitate the non–heat resistant and remaining high molecular weight proteins. MT in heat–treated cytosols was determined by differential pulse polarography (DPP) with a static mercury drop electrode (SMDE) using a 694 stand and a 693 processor (Metrohm). The electrode system consisted in a mercury capillary working electrode, an Ag/AgCl reference electrode and a platinum auxiliary electrode. The supporting electrolyte (1M NH_4Cl, 1M NH_4OH and 2mM $[Co(NH_3)_6]Cl_3$) was prepared weekly and stored at 4 ºC as described by Palecek and Pechan (1971). In absence of a commercial mollusc MT, Rabbit MT (forms I & II, from Sigma) was used for quantification of thiols using a standard–addition technique. The marked similarity between the

polarogrammes generated by rabitt and octopus MTs confirmed the suitability of using rabitt MT to calibrate the assay for octopus. The procedure followed Costa et al. (2008) methodology that was adapted from Bebianno and Langston (1989). Results are expressed as mg MT–equivalent g^{-1} tissue dry mass (dm).

Statistical analyses

Prior to statistical analyses, metal concentrations were tested for normality and equality of variances. Non-compliance with parametric ANOVA assumptions led to employment of the Kruskal-Wallis H (KW-H) and Mann-Whitney (U) non-parametric tests were used to evaluate the existing differences between metal and MT concentrations of individuals from A, B and C areas and between tissues. Principal Component Analysis (PCA) was applied to the three areas in each tissue in order to determine metals and MT associations. The significance for statistical analyses used was always $p<0.05$. The statistical analyses were performed using STATISTICA (Statsoft).

Results

The size and mass of octopuses sampled at areas A, B and C ranged in the following intervals: 146-165, 120-195 and 165-205 mm; 1162-1399, 805-2440 and 1231-1957 g, respectively. The proportion female:male was also similar in the three areas: 3:3 (A), 5:6 (B) and 3:3 (C). Concentrations of V, Cr, Co, Ni, Zn, Cu, As and Cd and MT in digestive gland, gills, kidney and gonads of *O. vulgaris* from the three areas of capture showed no significant differences (KW-H, $p<0.05$) with size/weight and gender of the analysed individuals. Therefore concentrations of octopus from each area were treated together.

Metal concentrations in tissues

The median, the percentile 25% and 75%, minimum and maximum concentrations of Co, Zn, Cu, Cd (logarithmic scale) and V, Cr, Ni and As (linear scale) in digestive gland, gills, kidney and gonads of *O. vulgaris* captured in the three areas are showed in figures 3.4.2 and 3.4.3, respectively. The essential elements Zn and Cu were the most abundant ones, medians varying several orders of magnitude: Zn from 71 μg g^{-1} in gills to 48050 μg g^{-1} in digestive gland; and Cu between 12 μg g^{-1} in gonads and 4200 μg g^{-1} in digestive gland. The medians of the other elements decreased from 50 to 0.25 μg g^{-1} in the following order: As> V> Cd> Ni> Cr> Co.

Digestive gland exhibited significantly (U, $p<0.05$) higher concentrations of Zn, Cd, V and Co than the other analysed tissues. Nickel and As were more abundant in kidney differing significantly (U, $p<0.05$) from the other three tissues. Arsenic was significantly (U, $p<0.05$) lower in digestive gland. Although being considered an essential element, Zn concentrations in all the analysed tissues were significantly (U, $p<0.05$) higher in area B than in A and C. Levels of Zn in area B ranged in the following intervals (μg g^{-1}): 12752-48051 in digestive gland, 726-912 in gills, 1026-7871 in kidney and 753-5585 in gonads. Cadmium concentrations (μg g^{-1}) showed significantly (U, $p<0.05$) higher levels in area A than in B and C for digestive

gland (140-555), gills (0.19-0.90), kidney (9.4-25) and gonads (0.072-0.35). Unlike Zn and Cd, concentrations of V and Ni in digestive gland, of Cu and Cr in gills and kidney, and Cu, Cr and Co in gonads showed no significant (KW-H, $p<0.05$) differences among the three areas.

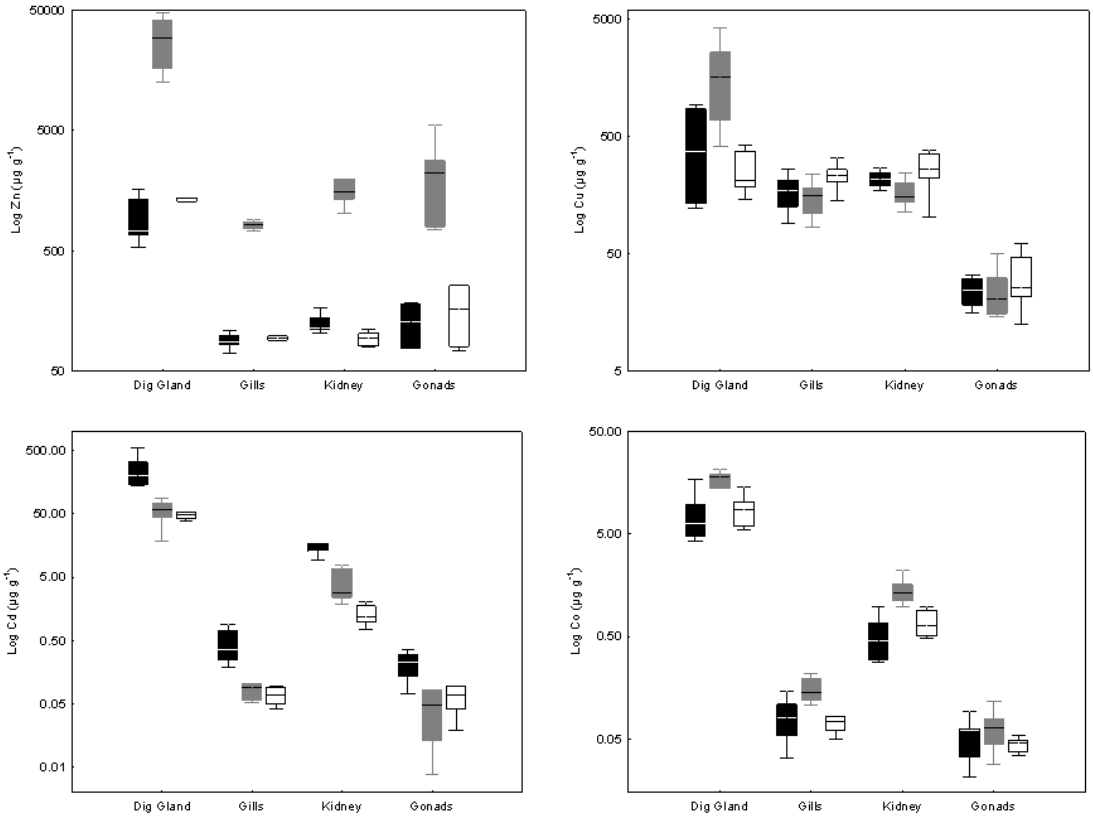

Figure 3.4.2 - Median, 25 and 75% percentile, minimum and maximum of Co, Zn, Cu and Cd logarithmic concentrations (µg g^{-1}, dry mass) in the digestive gland (Dig Gland), Gills, Kidney and Gonads of common octopus, *O. vulgaris* from areas A (black boxes), B (grey boxes) and C (white boxes).

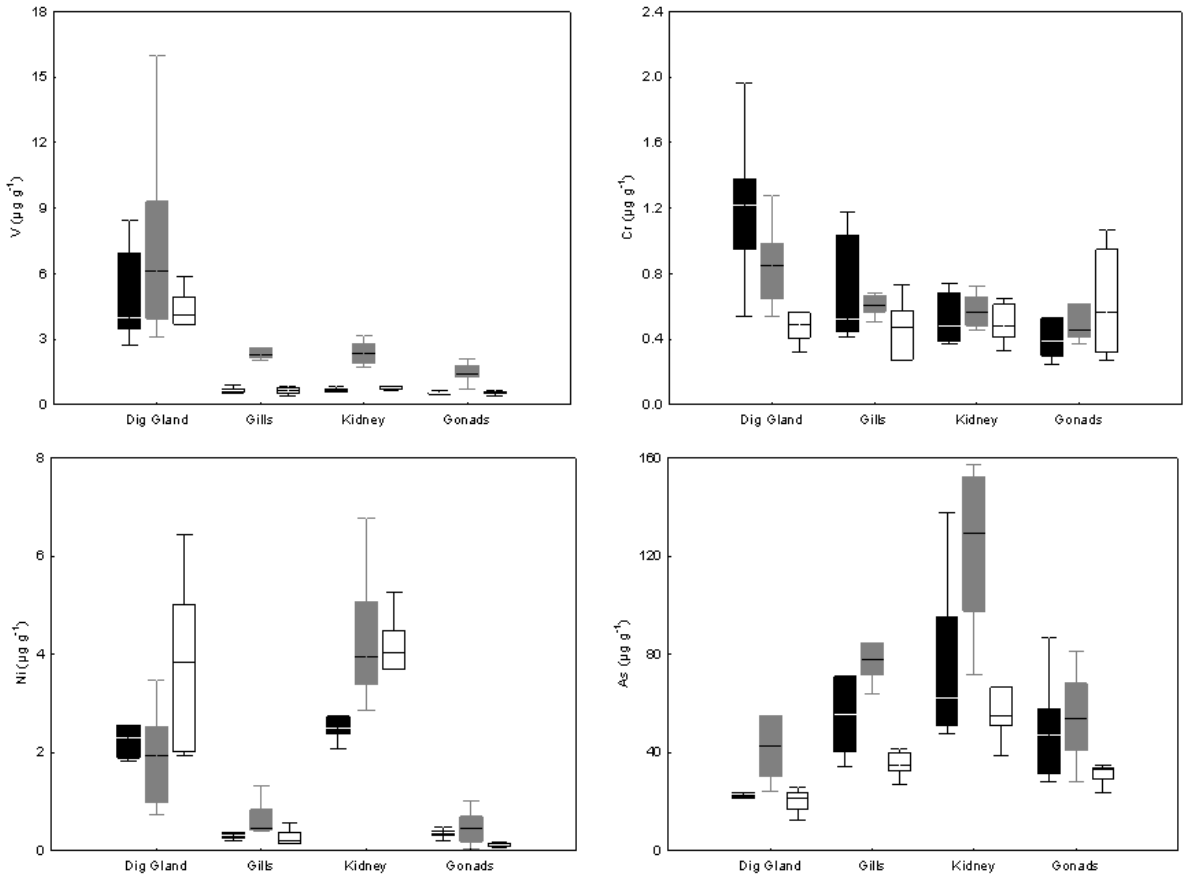

Figure 3.4.3 – Median, 25 and 75% percentile, minimum and maximum, of V, Cr, Ni and As concentrations (µg g^{-1}, dry mass) in the digestive gland (Dig Gland), Gills, Kidney and Gonads of common octopus, *O. vulgaris* from areas A (black boxes), B (grey boxes) and C (white boxes).

Levels of metallothioneins-like proteins (MT)

Figure 3.4.4 shows the median, the percentile 25% and 75%, minimum and maximum of MT concentrations in digestive gland, gills, kidney and gonads of *O. vulgaris* captured in the three areas. The median of MT (mg g^{-1}, dry mass) in the analysed tissues of octopus from these areas varied two orders of magnitude: between 0.10 in gills and 20 in digestive gland. Values in digestive gland were significantly (U, $p<0.05$) higher than in gills and kidney (area A) and gills, kidney and gonads (area B). In area C levels in digestive gland, kidney and gonads were significantly (U, $p<0.05$) higher than in gills. Concentrations of MT in digestive gland and gills of *O. vulgaris* from areas A and B were significantly (U, $p<0.05$) higher than from area C. No significant (KW-H, $p<0.05$) differences were found for kidney and gonads between the three areas.

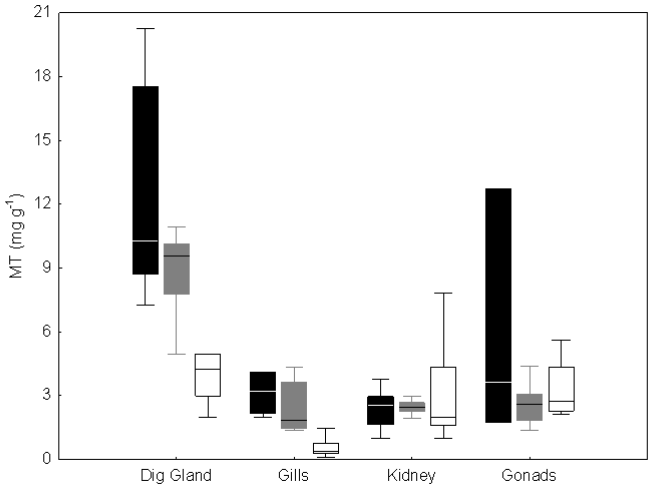

Figure 3.4.4 – Median, 25 and 75% percentile, minimum and maximum, of metallothionei (MT) concentrations (mg g^{-1}, dry mass) in the digestive gland (Dig Gland), Gills, Kidney and Gonads of common octopus, *O. vulgaris* from areas A (black boxes), B (grey boxes) and C (white boxes).

DISCUSSION

The effect of biological parameters (i.e., size, mass and gender) on the metal accumulation in cephalopods is far from being consensual (Miramand and Bentley, 1992; Rossi et al., 1993; Bustamante et al., 1998a; Seixas et al., 2005; Bustamante et al., 2006; Pierce et al., 2008). These discrepancies may probably result from the influence of other factors on the metal accumulation in cephalopods.

The metal concentrations found in the current work corroborates previous studies evidencing the ability of *O. vulgaris* to accumulate high levels of essential (Zn, Cu) and non-essential elements (e.g., Cd) in digestive gland (e.g., Miramand and Guary, 1980; Raimundo et al., 2004, 2005; Napoleão et al., 2005). However, Zn, Cu and Cd found in digestive gland reached remarkable elevated levels (max. 48050, 4200 and 555 µg g^{-1}, respectively) exceeding largely values reported in other studies with this octopus species (Miramand and Guary, 1980; Soldevilla, 1987; Raimundo et al., 2004; Napoleão et al., 2005; Raimundo et al., 2008). Zinc concentrations in gills, kidney and gonads of specimens from area B also surpassed levels reported in other works. Vanadium levels in the digestive gland of octopus collected in the current work were comparable to the ones reported by Seixas and Pierce (2005) for the same species. Comparison of other trace elements is not possible due to the lack of available data in the literature. To the best of our knowledge no information on MT has been published for *O. vulgaris* and this work is therefore a novelty. The obtained MT levels in digestive gland were higher than in the octopus *E. cirrhosa* and comparable to values found in the squid *L. vulgaris* (Bustamante et al., 2002).

Relation between MT and Metals

When one search relationships between MT and trace elements accumulated in the analyzed octopus tissues, the first perception is that MT variation (Figure 3.4.4) does not correspond to the broad differences of metal concentrations between digestive gland and other tissues, and differences between areas (Figures 3.4.2 and 3.4.3). Although other variables may influence the MT production, such as temperature (Phillips and Rainbow, 1994), the plausible reason for the obtained narrow variation of MT is that not all accumulated metals are in forms or present at sufficient levels to induce the MT transcription.

The levels of MT in digestive gland of octopus from areas A and B are significantly ($p<0.05$) higher than values found in area C (Figure 3.4.4). Furthermore, those concentrations reached one order of magnitude above the levels registered in wild bivalves where MT was considered to be induced by the presence of metals in their tissues (Bustamante, 1998; Amiard et al., 1998; Raspor et al., 1999; Geffard et al., 2002; Smaoui-Damak et al., 2004). The elevated levels of MT in digestive gland of octopus from those two areas may be hypothesised as a response to high metal accumulation, as the consequence of being exposed to a more contaminated environment (Caetano and Vale, 2003; Raimundo et al., 2004). Levels of MT in gills also differed between areas in a similar manner, but levels are much lower than in digestive gland. These results are in line with works with *Ruditapes decussatus* and *Cerastoderma glaucum* pointing that MT from digestive gland and gills give a more sensitive response to assess the effects of metal exposure (Bebianno et al., 2000; Machreki-Ajmi et al., 2008). This sensitivity could be due to the physiological roles of those organs. Digestive gland, which has a key function in the digestive process, is also recognized for its ability to store high metal levels (Martin and Flegal, 1975; Miramand and Guary, 1980). Gills have been pointed as a pathway for metal uptake and short-time storage (Bebianno and Serafim, 2003; Machreki-Ajmi et al., 2008), although water as less influence in comparison to food.

Previous research on MT induction proved that several metals, at a certain level in tissue, may trigger the MT synthesis to sequester metallic ions and reduce their toxicity (Roesijadi, 1996). Moreover, the association of MT with metals may vary in time as elements with stronger affinity to MT become available in the tissue (Roesijadi, 1996; Pourang et al., 2004). In an attempt to identify which element or elements may be the major contributors for the elevated MT in digestive gland and gills, a principal component analysis (PCA) were applied to data of each tissue (Fig. 3.4.5). The variance explained by the two principal factors of PCA varied between 45% (digestive gland) and 60% (gills). In these tissues MT showed a preferential association with Cd and Cr. Good separation was obtained for the three areas of capture, being MT, Cd and Cr closer associated with samples from area A. The results from PCA reflect the significantly ($p<0.05$) higher levels of Cd in digestive gland and gills of specimens captured in area A (Fig. 3.4.2) and indicates an additional Cr-MT linkage. The relationship between these two variables has been reported in laboratory studies with various molluscs exposed to Cd (Bebianno et al., 2000; Bebianno and Serafim, 2003; Ng et al., 2007; Machreki-Ajmi et al., 2008). The Cr-MT association was more pronounced in gills, pointing that the soluble Cr^{6+} maybe a preferential trigger for MT production. That association has been already observed in gills and liver of the rainbow trout exposed to Cr (Roberts and Oris, 2004) and in

liver of mice and chick (Fleet et al., 1990; Ohta et al., 1993). The reduction of Cr^{6+} to Cr^{3+} generates reactive oxygen species (ROS) leading to increased oxidative stress (Travacio et al., 2000), which is believed to induce the synthesis of MT (Thornalley and Vasak, 1985). Despite the high levels of Zn in digestive gland and gills of specimens from area B and the reported affinity of this element to MT (Park et al., 2001; Pourang et al., 2004), the PCA showed a lack of association of this element with MT. Due to the affinity of MT to various elements, it should not be excluded the possibility of Cd replaced Zn in Zn-MT linkage (Machreki-Ajmi et al., 2008) as Cd is incorporated in digestive gland and gills. Similar mechanism has been reported to clams exposed to a sequence of metals (Zn, Cu and Cd) (Bebianno and Serafim, 2003).

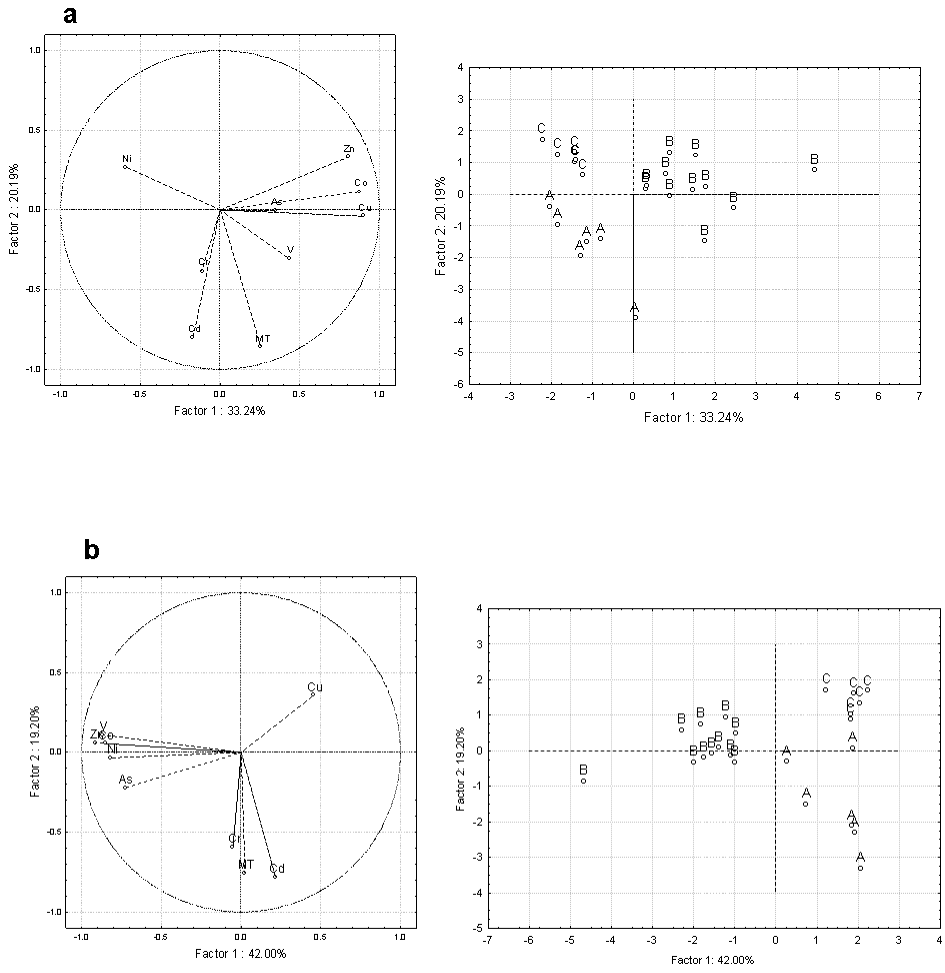

Figure 3.4.5 – Principal component analysis of metals and metallothionein (MT) concentrations in the (a) digestive gland (Dig Gland), (b) Gills, (c) Kidney and (d) Gonads of common octopus, *O. vulgaris* from areas A, B and C.

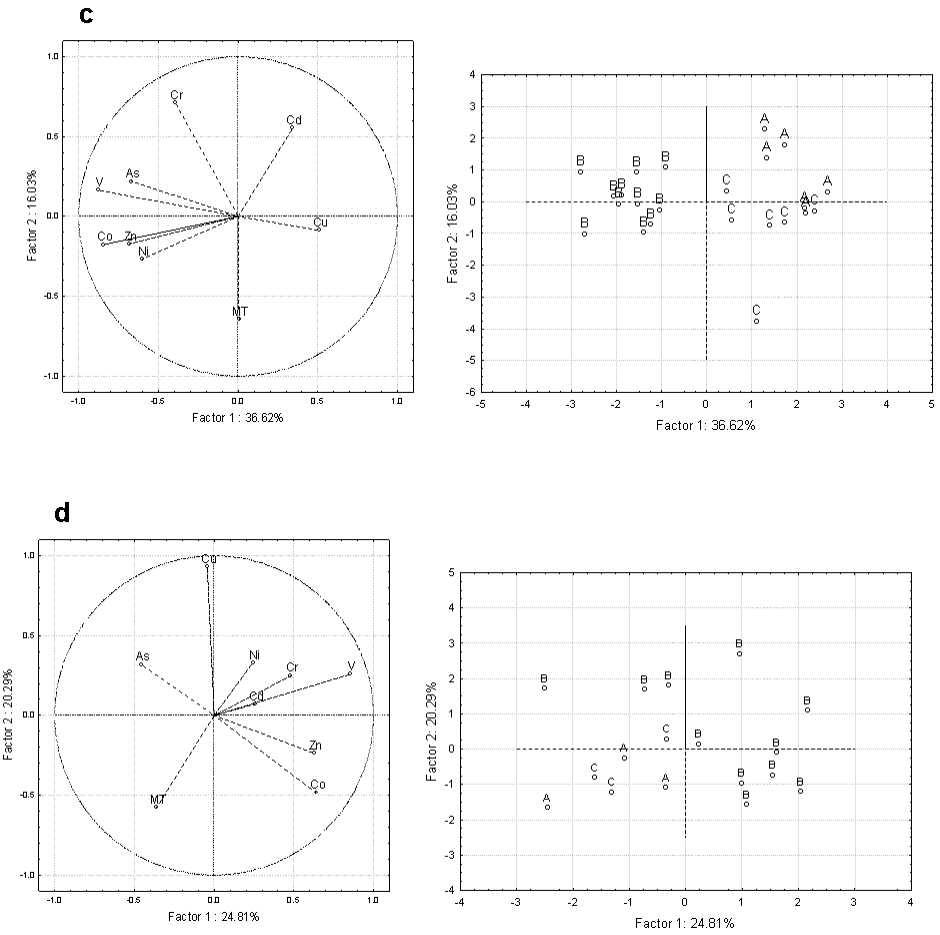

Figure 3.4.5 (Continued) - Principal component analysis of metals and metallothionein (MT) concentrations in the (a) digestive gland (Dig Gland), (b) Gills, (c) Kidney and (d) Gonads of common octopus, *O. vulgaris* from areas A, B and C.

Kidney presented higher levels of Co, Ni, As and Cd than gills and gonads, and in the case of As surpassing the levels in digestive gland (Figures 3.4.2 and 3.4.3). However, the PCA applied to this tissue showed that MT points are projected in opposite quadrants to metals (Figure 3.4.4). Likewise the MT levels in gonads had no correspondence to the variation of metal concentrations. These results point that MT levels in these tissues had no relations with accumulated metals.

The quantification of metallothioneins in digestive gland and gills suggests an important role of these proteins in the detoxification of Cd and Cr, and the sensitivity of those tissues to environmental conditions. In kidney and gonads the lack of relations with trace elements suggests that an alternative mechanism of detoxification may be present, and further studies are needed to characterise them. Although known to induce MT (Fleet et al., 1990; Albores et al., 1992; Park et al., 2001; Amiard et al., 2004, 2008), no relation was obtained with levels of Co, Ni and As in the analysed tissues of *O. vulgaris*, showing that the levels found for these metals may not be sufficient to induce MT or other defence

mechanism were involved. It was showed that high metal bioaccumulation in *O. vulgaris* may lead to a response involving metallothionein.

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Chapter 4

Genotoxic effects

Context

Once metals entered the cells they undoubtedly become bound to a variety of ligands. However, as levels surpass the capacity of detoxification systems to protect the cell, damages occur. Among the molecular components of the cell, DNA is an important target. The exposure of organisms to metal contamination promotes interactions between metals and DNA, changing the integrity of this molecule. It have been proposed that DNA may be a useful endpoint for assessing the effects of environmental pollutants at individual, population and ecosystem level

Summary

This chapter describes the DNA damages in digestive gland, kidney, gills and gonads of *Octopus vulgaris*, relating with metal accumulation and tissue function.

Chapter 4.1

DNA damage and metal accumulation in four tissues of feral *Octopus vulgaris* (Portugal)

Raimundo, J., Costa, P.M., Vale, C., Costa, M.H., Moura, I. (2010). DNA damage and metal accumulation in four tissues of feral Octopus vulgaris (Portugal). Mutation Research. Ecotoxicology and Environmental Safety 73, 1543-1547.

Abstract

The alkaline comet assay has been employed for the first time to estimate the basal DNA damage (SB) in the digestive gland, gills, kidney and gonads of *Octopus vulgaris.* Octopuses were captured in two coastal areas off the cities of Matosinhos (N) and Olhão (S), Portugal. The coast off Matosinhos is influenced by discharges of the Douro River, city of Porto, industries and intensive agriculture, while Olhão is an important fisheries port located in an area with strong Mediterranean influence. Previous works point to contrasting metal availability in the two coastal areas. Among the analyzed tissues digestive gland presented the highest levels of Zn, Cu, Cd and Pb. Tissues of specimens from Matosinhos presented high levels of Cd and from Olhão enhanced Pb concentrations. The SB in digestive gland, gills and kidney were more accentuated in specimens from Matosinhos than from Olhão, suggesting a stronger effect of contaminants, especially Cd. Elevated SB was registered in digestive gland, recognised for its ability to store and detoxify accumulated metals. The DNA damage in kidney, gills and gonads was lower, which is in accordance with reduced metal bioaccumulation. The broad variability of SB in the three tissues may mirror tissue function, specific defences to genotoxicants and cell-cycle turnover.

Introduction

Among the molecular components of the cell, DNA is an important target of environmental stress in organisms (Frenzilli et al., 2001). Various environmental contaminants are known mutagens. Damage to DNA may lead to mutations, strand breaks, altered bases (Shugart, 2000) and eventually carcinogenesis and other health disorders (Kurelec, 1993). It may result in severe consequences at individual, species and ecosystem level (Klobucar et al., 2003). Therefore DNA damage has been considered in toxicity testing.

The single–cell gel electrophoresis (Comet) assay has become a widespread technique for detection of DNA damage induced by xenobiotics (e.g. Cd, (Desai et al., 2006; Fourie et al., 2007); Hg, (Tran et al., 2007); organic compounds, (Costa et al., 2008)). The alkaline version of the assay has proven to be a simple and reliable method for the quantitation of total DNA fragmentation as a result of the formation of single– and double–strand breakage, xenobiotic–DNA adducts and alkali–labile sites (e.g. unstable altered nucleotides) (Singh et al., 1988). The Comet assay has been used in a wide range of aquatic organisms, such as marine diatoms (Desai et al., 2006), bivalve molluscs (e.g. Jha et al., 2005; Desai et al., 2006) and fish (e.g. Ahmad et al., 2006; Costa et al., 2008), for the biomonitoring of coastal environments. Most of studies deal with one and/or a limited number or combinations of contaminants, and thus research in aquatic ecosystems with complex mixtures and interactions of metals and other contaminants is still missing. Moreover, to our knowledge this technique has not been applied to assess DNA damage in cephalopod tissues.

The common octopus, *Octopus vulgaris*, is a sedentary cephalopod inhabiting coastal waters and thus susceptible to be exposed to contamination (Mangold, 1983). Local environmental conditions influence metal accumulation in tissues and several studies have proved the ability of these organisms to accumulate high levels of essential and non-essential elements, especially in digestive gland (e.g.

Miramand and Guary, 1980; Miramand and Bentley, 1992; Bustamante et al., 1998a, b; Raimundo et al., 2004, 2005; Napoleão et al., 2005). However, only few data exists regarding tissue-level effects of accumulation [Bustamante et al., 2002; Raimundo et al., 2008].

The aim of this study was to examine whether DNA strand breaks in digestive gland, gills, renal appendages (herein called kidney) and gonads of the common octopus, *Octopus vulgaris*, are related with the accumulation of Zn, Cu, Cd and Pb. This hypothesis was tested in feral animals captured in two areas of the Portuguese coast with contrasting availability of these elements.

Material and Methods

Samples

Twelve common octopuses, *Octopus vulgaris*, were collected from commercial catches in November 2007 in two coastal areas of Portugal: off Matosinhos (n=6) and off Olhão (n=6) (Figure 4.1.1). The Matosinhos coastal zone is drained by Douro, an important Iberian river. The Douro estuary is surrounded by the city of Porto and metropolitan area with industries and the riverine margins by intensive agriculture (Araújo et al., 2002). Toxicological studies were performed with fishes from the Douro estuary (e.g. Ferreira et al., 2006, 2008). However, a survey in the coastal area has reported high levels of Cd and Cu in the water column particularly in winter (Caetano and Vale, 2003) and slight enhancement of DDT compounds and PCBs (Quental et al., 2003). The southern zone (Olhão) is influenced by small rivers crossing the Iberian Pyritic Belt with ores containing large quantities of Zn, Cu and Pb, minor Cd content and traces of Ni (Palanques et al., 1995; Elbaz-Pulichet and Leblanc, 1996). This geological feature has been shown to affect Pb concentrations in octopus tissues (Raimundo et al., 2009). The sampled organisms were kept on ice until laboratory. Then each individual was weighted and mantle length and sex determined. The specimens were immediately dissected, digestive gland (without rupture of the outer membrane), gills, kidney and gonads of each organism being totally removed.

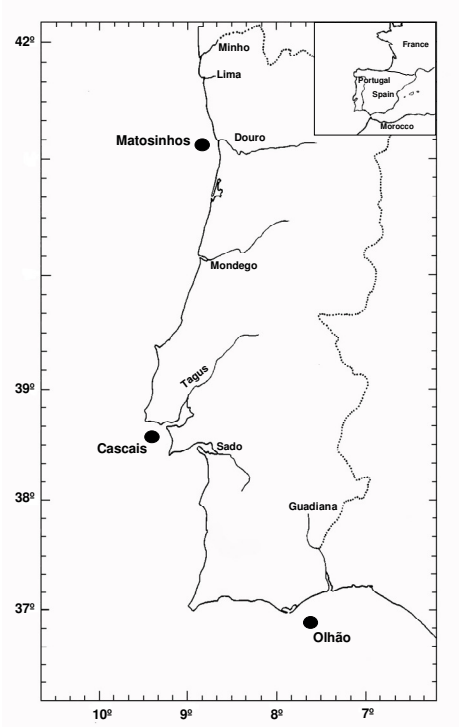

Figure 4.1.1 – Location of the two areas of capture of *Octopus vulgaris* in the Portuguese Coast: Matosinhos and Olhão.

Analytical methodology

Metals

Metals were analysed in lyophilised, grinded and homogenised samples after digestion 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 lab ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Three procedural blanks were prepared using the same analytical procedure and reagents, and included within each batch of samples. Concentrations of Zn, and in the case of digestive gland, Cu and Cd were determined by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100) and Cu, Cd and Pb by a quadropole ICP-MS (Thermo Elemental, X-Series). The accuracy of these analytical methods was assessed by the analysis of international certificate standards (DORM-1, DORM-2 – dogfish muscle; DOLT-1 – Fish liver and TORT-1, TORT-2 – lobster hepatopancreas). The results obtained were in good agreement with the certified values ($p<0.05$). Procedural blanks always accounted for less than 1% of the total metal in the samples. All the results are given as medians and ranges in micro gram per gram of dry mass tissue (μg g^{-1}; dm).

DNA Strand Breaks

DNA total strand breakage (DNA-SB) was assessed through the alkaline single–cell gel electrophoresis (Comet) assay (Singh et al., 1988) and adapted from the method described by Costa et al. (2008). Aliquots of fresh digestive gland, gill, kidney and gonad cells were ressuspended in KSS (Kenny's Salt Solution) in the proportion 1:1 (w/v), and placed on slides pre-coated with 1% (w/v) normal melting-

point agarose in TAE buffer (the slides were allowed to dry for at least 48 h) and covered with a coverslip. After agarose solidification (15min, 4 ºC) the coverslip was removed and the slides were dipped for 1 h at 4 ºC in lysis solution (2.64% NaCl (w/v), 3.72% EDTA (w/v) and 5mM TRIS) to which was added 10% (v/v) DMSO and 1% (v/v) Triton-X 100 just before use. Slides were afterwards placed in cold (4 ºC) electrophoresis solution (pH 13) for 40 min to allow DNA-unwinding and enhanced expression of alkali-labile sites. Electrophoresis was for 30 min at 25 V, in the cold (4 ºC), using a Sub-Cell model 96 apparatus (Bio-Rad). Slides were afterwards neutralized in 0.1M Tris–HCl buffer (pH 7.5) for 15 min. All preparatory steps were performed under controlled temperature (≈16 ºC) to avoid gel lifting from the slides and all solutions and electrophoresis apparatus were kept in the dark and in the cold to minimize accessory DNA degradation. Approximately 100 comets were analysed per slide after staining with 0.02mg mL^{-1} ethidium bromide (EtBr). Comets were analysed using the CometScore (TriTek). The percentage DNA in the tail was employed as a direct measure of DNA-SB (Lee and Steinert, 2003). DMLB microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps was used, equipped with an N2.1 filter, all from Leica Microsystems. The comet assay was successfully employed in all surveyed tissues, as indicated by the retrieving well-defined nucleoids as well as damaged cells (Fig. 4.1.2).

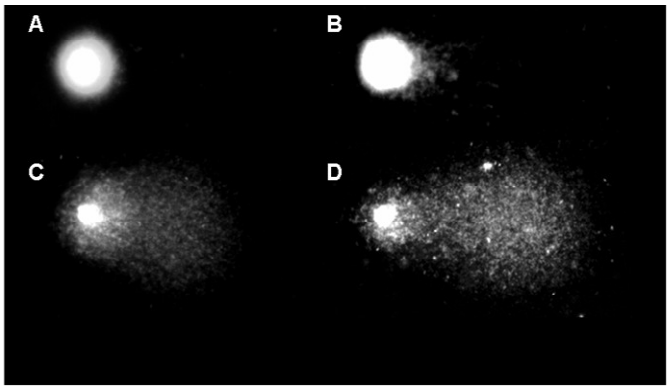

Figure 4.1.2 – Comet examples of DNA-SB from *Octopus vulgaris*: ≅ 0% (A, gonads), ≅ 27% (B, kidney), ≅ 68% (C, gills) and ≅74% (D, digestive gland).

Statistical analysis

Prior to statistical analyses, metal concentrations were tested for normality and equality of variances. Non-compliance with parametric ANOVA assumptions led to employment of the Kruskal-Wallis H (KW-H) and Mann-Whitney (U) non-parametric tests were used to evaluate the existing differences between metal concentrations and DNA fragmentation of individuals from the study areas and between tissues. The significance for statistical analyses used was always $\alpha = 0.05$. Statistical analyses were performed using Statistica (Statsoft).

Results

Influence of size/weight and gender

Concentrations of Zn, Cu, Cd and Pb in digestive gland, gills, kidney and gonads showed no significant (U, $p>0.05$) differences with the size/weight of the captured octopus. The DNA stand breaks presented also a lack of relationships with those two biological parameters. Levels of Cd and Pb showed no significant differences (U, $p>0.05$) with the gender. However, Cu concentration was more accentuated in digestive gland of males (KW-H=8.3, p=0.04) and in female gonads (KW-H=8.3, p=0.004), and Zn levels in male gonads (KW-H=4.3, p=0.004). The DNA total strand breaks was higher in gonads of females than of males (KW-H=6.8, p=0.009).

Metal partitioning

Figure 4.1.3 shows the median, the percentile 25% and 75%, and maximum and minimum of Zn, Cu, Cd and Pb concentrations (μg g^{-1}, dm) in digestive gland, gills, kidney and gonads of *O. vulgaris* from each area of capture. The most noticeable aspect consist of several orders of magnitude difference of Zn, Cu, Cd and Pb levels between digestive gland and other analysed parts. The contrast was less pronounced for the essential elements Zn (2626 to 71 μg g^{-1} in gills) and Cu (931 and 12 μg g^{-1} in gonads) than for Cd (556 to 0.019 μg g^{-1} in gonads) and Pb (10 to 0.057 μg g^{-1} in gonads).

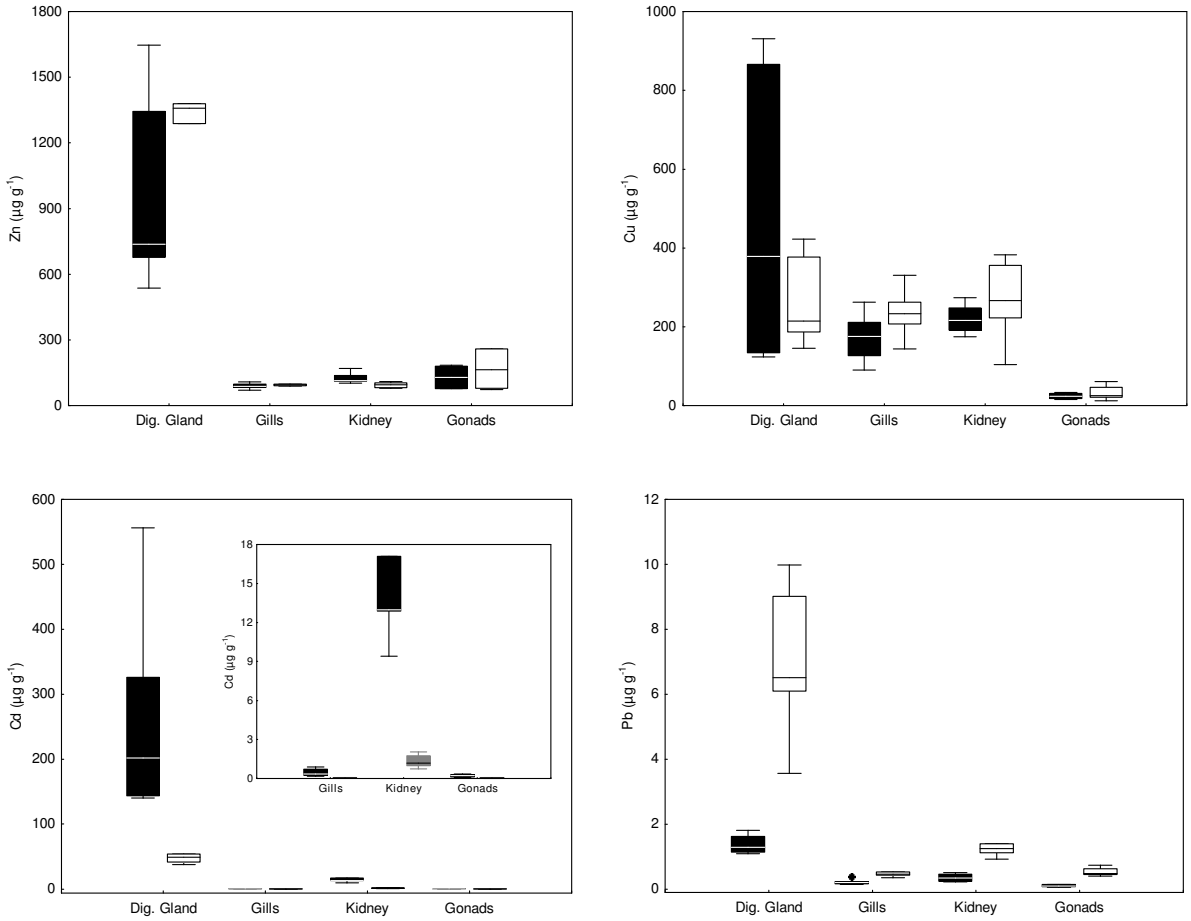

Figure 4.1.3 - Median, 25 and 75% percentile, minimum and maximum, and the extreme values (□) and outliers (•), of Zn, Cu, Cd and Pb concentrations (µg g^{-1}, dry weight) in the digestive gland (Dig Gland), Gills, Kidney and Gonads of common octopus, *O. vulgaris* from Matosinhos (black boxes) and Olhão (white boxes).

Differences of metal concentrations between areas of capture

Regardless the observed differences of Cu and Zn concentrations with the gender, in digestive gland and gonads, the comparison between the two areas of capture was considered viable because each set of samples contained equal number of males and females. Despite the contrasting accumulation of metals in octopus tissues, all the analyzed tissues of specimens from Matosinhos presented higher levels of Cd ($p<0.05$) than those from Olhão. Differences reached one order of magnitude in digestive glands. In contrast, enhanced Pb concentrations ($p<0.05$) were found in individuals from Olhão for all analyzed tissues. The differences were also more marked in digestive gland. Similar intervals of Zn and Cu levels were registered in specimens from both areas (KW-H, $p>0.05$).

DNA strand breakage in tissues

Like for metal concentrations it was assumed that the equal number of males and females analysed implies that gender has the same weight in the DNA integrity of tissues from octopus of the two areas of capture. Figure 4.1.4 shows the median, the percentile 25% and 75%, and maximum and minimum of the percentage of DNA strand breaks (SB) in digestive gland, gills, kidney and gonads of specimens from the two areas independently of the gender. Likewise accumulated Zn, Cu, Cd and Pb, the SB in digestive gland exceeded significantly (KW-H=14, p=0.003 for Matosinhos and KW-H=13, p=0.005 for Olhão) the values registered in gills, kidney and gonads. The DNA SB in digestive gland, gills and kidney varied significantly between specimens from Matosinhos and Olhão, being the medians respectively: 86 and 73% (digestive gland); 71 and 16% (gills); and 47 and 31% (kidney). Conversely, medians of gonads found in the two areas were not statistically different: 16 and 33%. The tissue samples from Matosinhos exhibited higher individual variability of SB than from Olhão. Gills and gonads presented broader intervals of SB. Gender explains the differences registered in gonads. Males presented lower SB values than females: 12-25% and 42-55% (Matosinhos) and 3.2-4.0% and 27-34% (Olhão), respectively.

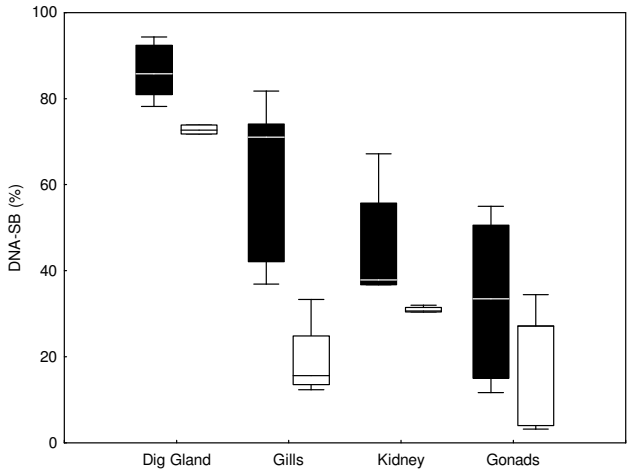

Figure 4.1.4 – Median, 25 and 75% percentile, minimum and maximum, and the extreme values (□) and outliers (•), of DNA strand breakage (DNA-SB) (%) in the digestive gland (Dig Gland), Gills, Kidney and Gonads of common octopus, *O. vulgaris* from Matosinhos (black boxes) and Olhão (white boxes).

Discussion

The results obtained in this work point to a negligible influence of size/weight on the partitioning of Zn, Cu, Cd and Pb among digestive gland, gills, kidney and gonads of *O. vulgaris*. The effect of these biological parameters on the metal accumulation in cephalopods is far from being consensual (e.g. Miramand and Bentley, 1992; Bustamante et al., 1998a; Storelli and Marcotrigiano, 1999; Raimundo et al., 2004, 2005; Seixas et al., 2005). Instead, it appears that bioaccumulation processes in tissues interacting

with the environment is strongly influenced by metal availability. Indeed, significantly higher levels of Cd in all analysed tissues of octopus collected off Matosinhos, which corroborate results from previous works in the area (Raimundo et al., 2004, 2005; Napoleão et al., 2005; Seixas et al., 2005), are in line with enhanced Cd concentrations registered in coastal waters adjacent to the local estuaries (Caetano and Vale, 2003). Likewise, elevated Pb levels in octopus from Olhão were consistent with findings reported for specimens from this area (Raimundo et al., 2004; Napoleão et al., 2005). In addition, the ratios of stable lead isotopes in sediments and digestive gland of octopus showed a low radiogenic signature, which indicates a strong influence of natural sources related to the geological feature of the Iberian Pyrite Belt (Raimundo et al., 2009).

Most studies on DNA integrity searched whether tissue responds to toxic conditions in the environment or under laboratory experiments. To the best of our knowledge DNA damage has not been assessed in *O. vulgaris*, and thus reporting SB in digestive gland, kidney, gills and gonads of this species by means of the comet assay is a novelty. The DNA damage registered in digestive gland, kidney and gills of octopus were statistically higher in specimens sampled off Matosinhos than Olhão. Since the study was performed in two natural environments, the wild specimens were exposed to the mixture of contaminants present in each coastal area. Under these conditions it is thus difficult to associate tissue responses to individual or collective toxic conditions. Zinc and Cu showed no significant differences on tissue accumulation between the areas and due to their role on essential biological processes it is difficult to predict any response in the analysed tissues. Conversely, the accumulated Cd and Pb presented a pronounced contrast between the two coastal areas and levels in certain tissues point them as probable genotoxicants. The geographical differences obtained for the DNA strand breaks are in line with the Cd distribution, suggesting that DNA damages should be stronger induced by Cd than Pb. This hypothesis is supported by experiments with other molluscs pointing the effect of Cd on DNA integrity. For example, the marine crab, *Charybdis japonica*, showed a positive dose response between DNA damage levels and concentrations of Cd in tissues (Pan and Zhang, 2006). Mussels exposed to a mixture of genotoxicants have shown to induce DNA alterations (e.g. Burgeot et al., 1996; Boelsterli, 2003). The broad variation of DNA strand breaks recorded in all tissues of specimens that accumulated higher quantities of Cd (Matosinhos) is in accordance with this hypothesis. In spite of the small number of sampled individuals, the consistently high variability observed in all analysed tissues suggests either ongoing damage or set off of the repair mechanisms if the threshold value was reached (Black et al., 1996). Works on Pb genotoxicity in fish have shown that Pb(II) significantly increases DNA damage inactivating or altering the repair mechanisms (Obe et al., 2002; Ferraro et al., 2004). Nevertheless, other works pointed to less convincing relations between Pb contamination and DNA damage, invoking complex interactions between inorganic Pb and protein kinase (Boelsterli, 2003; Ramsdorf et al., 2009).

Regardless of the metals available in each sampled area, the DNA damage was more accentuated in digestive gland than in the other analyzed tissues (Figure 4.1.3). This enhancement is in accordance with the differences of accumulated metals between tissues (Figure 4.1.2). However, the intervals of DNA

strand breaks among tissues were far narrower than the 3-4 orders of magnitude recorded for Zn, Cd and Pb concentrations. The most plausible explanation for this disparity is that only a fraction of metals present in the digestive gland interferes with DNA repair processes and enhances genotoxicity. Presumably, it reflects the existence of mechanisms to store and detoxify metals (e.g., Bustamante et al., 2002), including association with sensitive cellular components such as, organelles and enzymes (Wallace et al., 2003). Gills and kidney of octopus from Matosinhos presented a broader variability on the DNA damage than digestive gland, although such correspondence was not registered in the accumulated metals. The branchial epithelium represents the primary target for water-borne contaminants. Pan and Zhang (2006) showed that the direct and continuous contact of contaminants with crab gill interferes with its functions (i.e. ionic balance and gas exchange) due to ongoing damage. Alternatively, the variability of DNA damage found in kidney may be the result of its excretory function leading to metal elimination (Rainbow and Phillips, 1993). The continuous exposure of these tissues to contaminants may therefore explain the variability on the DNA damage. The DNA damage as a biomarker of exposure to contaminants was not evident in gonads, since the larger differences were found between males and females in both areas. Presumably, the lower DNA fragmentation in the male's gonads reflects the higher rate of germ-cell division in testis (Boyle and Rodhouse, 2006).

Although digestive gland is recognised as a xenobiotic storage and detoxifying organ, DNA damage reached high levels, particularly in specimens accumulating high concentrations of Cd. Lower DNA damages in other tissues like kidney, gills and gonads are in accordance with the lower accumulated metals although the broad variability between individuals may reflect tissue function and cell-cycle turnover. It is clear that different tissues have different sensitivities to genotoxicants, likely due to differential mechanisms of defence and DNA repair.

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Chapter 5

General discussion

Context

This chapter presents a general discussion on the work developed in this thesis. This discussion consolidates the outcomes of the work presented in Chapters 2 to 4. The first part describes the three main questions raised in this work. The second part discusses the several results obtained and the most important conclusions for each. And the third part presents the final remarks.

General discussion

The works presented in this thesis were carried out with wild octopus, *Octopus vulgaris*, from the local fishermen operating in three coastal areas in Portugal: Matosinhos, Cascais and Olhão. The organisms in these areas are exposed to different environmental conditions: Matosinhos and Cascais, are influenced by two of the major rivers in Portugal, Douro and Tagus, respectively, that receives domestic effluents, discharges from industrial activities in the regions and diffuse inputs from agricultural practices in the extensive drainage basins. Olhão is located in the southern coast far from important industrial centres. Although using wild octopus in the studies, specimens were chosen in a narrow range of sizes/weights and a similar proportion male:female. The selection of these individuals was an attempt to minimise the influence of those major biological factors on the bioaccumulation processes. However, environmental and other biological factors were not controlled as in planned laboratory experiments and therefore interpretation of key questions may be masked by synergetic processes in response to different pressures. Nevertheless these studies provide a more realistic image of metal accumulation, regulation and responses of wild organisms. The works included in this thesis aim to respond to three relevant questions:

1. Does *Octopus vulgaris*, exposed naturally to the availability of trace elements in three areas of the Portuguese coast, display differences on the accumulation and partitioning levels in tissues?
2. Do accumulated trace elements in octopuses induce sub-cellular responses in digestive gland, kidney, gills and gonads?
3. Do tissues accumulating elevated levels of trace elements exhibited evidence of genotoxicity?

Elemental concentrations and partitioning

The first step of this thesis was to establish which tissue preferentially accumulates high levels of contaminants (Chapter 2.1) providing data for the following studies (Chapter 2.2, 2.3, 3 and 4). Metal concentrations differed considerably among the eleven analysed tissues/organs of octopus (digestive gland, posterior salivary glands, kidneys, gills, gonads, branchial hearts, ink sac, stomach, skin, mantle and arm). Digestive gland exhibited the highest metal concentrations being, in general, one order of magnitude above those obtained in the remaining tissues. Some exceptions were observed, being Fe, Cu (Chapter 2.1), Ni, Cr and As concentrations (Chapter 3.3) similar or lower than levels observed in other tissues, e.g. gills, kidney, gonads and branchial hearts. The elevated levels obtained in the digestive gland of octopus are in line with other studies with cephalopods and corroborate the presence of efficient mechanisms to store metals in this organ (e.g. Miramand and Bentley, 1992; Bustamante et al., 2002; Raimundo et al., 2004, 2005; Napoleão et al., 2005; Seixas et al., 2005; Pereira et al., 2009). In octopus, digestive gland, as well as, kidney and gills, are potential indicators of the environmental availability of metals. Water surveys in the Portuguese coast, registered enhanced levels of Cd in the northern coast (Caetano e Vale, 2003) and a similar pattern was found in octopus tissues.

Like observed for Cd, other non-essential elements, such as Pb and Hg, also presented elevated concentrations in the digestive gland. The geographical differences observed between the three areas of study appear to suggest that accumulated levels respond to the increasing availability in the environment (Chapter 2.2 and 2.3).

For example total Pb concentrations in the digestive gland of octopus presented a considerable contrast between two areas of the Portuguese coast: Matosinhos (low levels) and Olhão (high levels). To the best of our knowledge for the first time, Pb stables isotopes were analysed in octopus tissue (Chapter 2.2). This initiative was done taking into account the success of using stable Pb isotopes in sediments to trace the origin of this element (Gobeil et al., 2001; Komárek et al., 2008). Isotopic ratios were determined in digestive gland of octopus and compared to the ones registered in the sediments from the same areas. An agreement was obtained between isotopic ratios in biological and sediment samples. This parallelism suggests that octopus reflect the sources of the Pb that is present in a specific environment. The less radiogenic signature obtained in digestive gland of octopus from Olhão (corroborated by findings in the sediments) suggested that elevated Pb levels found in that area are mainly from natural sources (Iberian Pyrite Belt). On the other hand, the lower total Pb concentrations and higher radiogenic signature observed in octopus from Matosinhos results from various origins, such as industrial influents. The contrasting aspect of these results is quite interesting and can be interpreted from different angles. It indicates that in a region with minor sources of industrial activities, like the southeast of Portugal, the lead present in coastal sediments is mainly derived from erosion or weathering of the drainage basin. Despite the distance of the Iberian Pyrite Belt, one of the largest mineral resources in Europe, it expands its influence until the coast. The most surprising aspect is the linkage with the biology. The relationship between signature of the accumulated levels in octopus digestive gland and in sediment provides a promising approach to the scope of bioaccumulation studies. In the light of these findings, Pb isotopic signature in the digestive gland of octopus seems to be a useful tool to distinguish and identify octopus populations.

Levels of Hg, Se and, for the first time MeHg, were determined in digestive gland and mantle of octopus from three study areas, Matosinhos, Cascais and Olhão (Chapter 2.3). Mercury, NeHg and Se were preferentially accumulated in the digestive gland, and the proportion of MeHg to total Hg was higher in mantle than in digestive gland. Moreover, concentrations found in octopus tissues were in accordance with levels reported for the surroundings of abovementioned areas (INAG; Canário et al., 2007). A proportional increase in both tissues for Hg and MeHg levels was observed in the less contaminated samples (specimens from Matosinhos and Cascais), suggesting that as MeHg enters the digestive gland and mechanisms of transport and storage in mantle may be activated. This can be further observed by a better relationship found between Hg and MeHg in mantle in comparison to digestive gland. However, after a threshold level (specimens from Olhão) the transport mechanisms between digestive gland and mantle may be less efficient. Another novelty of this work was the possible involvement of Se in the detoxification processes of Hg in digestive gland. The results obtained with

octopus are in line with previous studies on the interaction mechanisms between Se and Hg (Chen et al., 2001; Belzile et al., 2006). This result was more pronounced in individuals from the more contaminated area. The hypothesis of demethylation processes occurring in digestive gland of octopus is in line with a study with other cephalopod species by Bustamante et al. (2006).

It is noteworthy that although elevated metal levels (e.g. Cd, Pb and Hg) are found in digestive gland, octopus does not seem to evidence toxicity symptoms. Thus, efficient responses to metal accumulation must be present at the sub-cellular level.

Sub-cellular responses to elemental concentrations

The second step of this thesis was to search for sub-cellular responses to the elevated metal levels found especially in digestive gland (Chapter 3) providing information for possible genotoxic effects (Chapter 4).

Octopus vulgaris were collected in Matosinhos and Olhão in order to evaluate the sub-cellular partitioning (nuclei, mitochondria, lysosomes and microsomes) of Zn, Cu, Cd and Pb in digestive gland. This study was carried out since the association of metal to metal-sensitive sites, like organelles and enzymes, is often associated with detoxification mechanisms (Simkiss and Taylor, 1982; Phillips and Rainbow, 1989; Bustamante et al., 2002). Only a minor fraction (<7%) of the total Zn, Cu, Cd and Pb content was associated with the insoluble fractions (organelles), indicating that the large majority of these elements are trapped in the cytosol. The association with cytosolic proteins is in line with findings by Tanaka et al. (1983), Finger and smith (1987) and Bustamante et al. (2002; 2006). Interesting, it was found that although small percentages of metals were associated with the organelles, linear relations were obtained for concentrations in each organelle (nuclei, mitochondria and lysosomes) and in the whole tissue. Furthermore, this response was more evident in the organisms exposed to higher environmental levels, such as Cd in Matosinhos and Pb in Olhão. It seems that mechanisms of detoxification existing in the cytosolic fraction were not fully efficient to retain the contaminants, particularly for non-essential elements. Moreover, the lack of relations in some organelles suggests an absence of toxicity in the digestive gland of octopus from both areas.

Another two additional questions emerge from these results: Do other detoxifying and storage tissues (e.g. kidney and gills) respond in the same manner as the digestive gland? And what are the affinity/partitioning of other potentially toxic elements? Since the majority of the metal content was associated with cytosolic proteins, a different approach was used. Four “particulate” fractions were analysed: granules, mitochondrias, lysosomes plus microsomes and heat-denaturable proteins (HDP) containing enzymes and other non-enzymatic proteins, and one “cytosolic” fraction consisting of heat-stable proteins (HSP), including metallothioneins (MTs) and glutathione. The HSP fraction contained higher percentages of trace elements in the three tissues, although varying with element. This high association may be linked to the presence of proteins such as MTs, known for its metal affinity. This result suggests a possible mechanism of trace element detoxification preventing elements from reaching more

sensitive sub-cellular fractions (such as, organelles and HDP fraction). It as been registered the interaction of MT and Cu was in digestive gland of cephalopods (Bustamante et al., 2006). Good relations were obtained between total concentrations in digestive gland, kidney and gills and organelles and HSP for Cd and Co. Arsenic and Pb also presented good tendencies. These relations seem to be similar in digestive gland, kidney and gills, indicating that the role of the elements in the cells, and consequently their association with the sub-cellular fraction, may superimpose the response existing as a function of availability in the whole tissue.

In order to further investigate the preferential of elements for the cytosolic fractions, protein purification and element associations were attempted in octopus digestive gland. Octopus from two areas (Matosinhos and Olhão) with contrasting levels of non-essential elements, Cd and Pb, were analysed. Three patterns were obtained: Zn was associated with high and low molecular weight proteins (HMWP and LMWP); Cu and Cd preferentially associated with LMWP, with a small increment in the HMWP fractions; and Pb entirely associated with HMWP. A difference was observed in Cd distribution in organisms from Matosinhos, with a more pronounced peak obtained in the HMWP. This disparity may result from an enhancement of Cd levels in the digestive gland of specimens from this area suggesting a different mechanism of retaining Cd when levels are higher. It is known that Cd may substitute essential elements in proteins (Temara et al., 1997). Good correlation between Cd-Zn and Cd-Cu were found in the LMWP fractions, indicating the interference of these elements at a cellular level.

For the first time, MTs were quantified in octopus tissues (digestive gland, gills, kidney and gonads). The detected quantities is of crucial importance due to its importance as a detoxification mechanism in marine invertebrates (e.g. Bebianno and Langston, 1991; Viarengo and Nott, 1993). The elevated metal levels observed in octopus could be an important inducer of MTs that would prevent potential toxicity/damages. Metal-MTs associations were searched in octopuses collected in the three study areas. Digestive gland, gills, kidney and gonads respond to the availability of metals in the three environmental areas. Two main associations were discerned by the PCA analysis, Cd-MT and Cr-MT in digestive gland and gills. Moreover, the association Cd-MT in both tissues was closer associated (PCA) with samples from Matosinhos, which presented higher Cd concentrations. A lack of relations was observed for kidney and gonads suggesting the existence of alternative mechanisms of detoxification.

Genotoxic effects

Different detoxification mechanisms (organelles and MTs) were identified in octopus tissues. But the question is whether these mechanisms are sufficiently efficient to prevent effects. DNA was studied in octopus as a possible target for contaminants. For the first time, DNA strand breaks (SB) were analysed in octopus tissues, digestive gland, gills, kidney and gonads. Two contrasting areas were selected for this work, Matosinhos with high levels of Cd and Olhão with enhanced levels of Pb. All tissues respond with the same pattern to the differences in metals availability in the environment, and digestive gland presented the higher concentrations among tissues. Strand breaks in the digestive gland, known to have

the ability to store and detoxify metals, exceeded the values registered in the other analysed tissues. The broad variability of SB in gills, kidney and gonads may mirror tissue function and cell-cycle turnover. In addition, DNA damages in digestive gland, gills and kidney were more accentuated in organisms from Matosinhos than Olhão, suggesting a stronger influence of Cd in the induction of damages.

Final Remarks

This theses claim that metals accumulated in octopus tissues, namely in the digestive gland, respond to the availability existing in the environment and ingested food. Traditionally, these results imply that octopus is seen as a bioindicator of metal contamination. In addition to the uptake and storage of metals in digestive gland and other tissues, further responses at cellular level have been identified as well as genotoxicity effects. These combined results point that exposure to moderate of trace elements in the environment appear to lead to modification in the cellular systems of the organism. Further studies are needed to better understand the unusual ability of this species to accumulate such high metal levels as a detoxifying mechanism. Despite the high storage, namely of Cd, and effects at the cellular level there is no record of significant changes at population level. In fact, stocks of octopus in the Portuguese waters have not presented significant alterations in the last years (Pierce et al., 2010), and landings at Matosinhos and Olhão ports are major contributors to the national amounts. According to the Water Framework Directive (WFD), the lack of response at population level is insufficient to conclude about a pressure or contamination in the water masses close to the studied areas. The efficient mechanism of elimination/detoxification possessed by octopus or DNA damage is out of the scope of the WFD. Furthermore, because the levels of trace elements in edible parts of octopus are far below the limits of concern for human consumption, the responses and effects are not either considered a relevant matter in the perspective of the Marine Strategy Framework Directive. Although these Directives are important vehicles for the surveillance of the environmental status in order to a better protection of the marine environment, the cause-effect binary related to the contamination will only be explored in the case of changes at population level. This uncovered distance between early warning signals of stress in organisms and damages in population creates a knowledge gap that should be fulfilled.

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Appendix

Methodologies

Methodologies

Biological Samples

Samples of common octopuses, *Octopus vulgaris*, were collected from commercial catches landed in Matosinhos, Cascais and Olhão. Specimens were captured within two areas of 6 miles radius centred at each area.

Total body weight, mantle length (Figure 1) and gender were determined in each individual. Specimens were stored in individual plastic bags and frozen (-80 ºC) in order to minimize mobilization of metals among organs/tissues (Martin and Flegal, 1975). In the laboratory, tissues were totally removed under partially defrost conditions without rupture of the tissue, freeze-dried, grounded and homogenised.

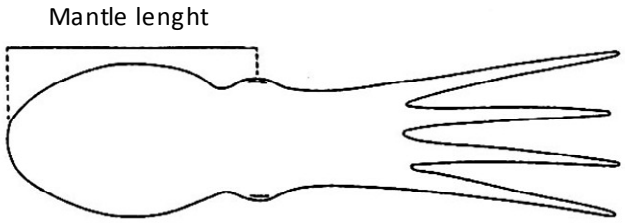

Figure Ap.1– Schematic representation of mantle length measurement.

Metal analyses

Biological samples. Samples (≈200 mg) were digested with a mixture of HNO_3 (sp, 65 % v/v) and H_2O_2 (sp, 30 % v/v) at 60 ºC for 12 hours, 100 ºC for 1 hour and 1 hour at 80 ºC according to the method described in Ferreira et al. (1990). Subsequently, the bombs content was poured into a 100-ml volumetric flask and filled up to 50 mL with Milli-Q water. Before ICP-MS analyses, must of the samples were diluted (2-5 times) with Milli-Q water.

Sediment samples. Two mineralization procedures were used for sediment samples: 1) digestion for Al quantification using HF (sp, 40 % v/v), Aqua Regia (HCl-36 %:HNO_3-65 %; 3:1). Subsequently, the bombs content was poured into a 100-ml volumetric flask containing 5.6 g of boric acid (H_3BO_4) and filled up with Milli-Q water as described by Rantala and Loring (1975); and 2) mineralization for analysis of Pb concentration and stable Pb isotopes by using the first step of the previous method, without the boric acid, evaporated to near dryness and elute with HNO_3 (double-distilled) and Milli-Q water (18.2 MΩ.cm) (Caetano et al., 2007). After digestion samples were poured into a 100-ml volumetric and filled up with Milli-Q water.

Procedural blanks and reference material were prepared using the same analytical procedures and reagents, and included within each batch of 10 samples.

Analytical methods. Metals with higher concentrations were determined using a flame atomic absorption spectrometry (Perkin Elmer AA100) with air-acetylene flame and concentrations determined with the standard addition method. The remaining elements were determined using a quadropole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. A 7-points calibration within a range of 1 to 100 µg L^{-1} was used to quantify total elements concentration. The precision and accuracy of the elemental concentration measurements were determined through repeated analysis of references materials (for organisms and sediments), using ^{115}In as internal standard. Variability was lower than 2 %. Procedural blanks always accounted for less than 1 % of the elements in the samples.

For Pb isotope determinations, between every two samples, corrections for mass fractionation were applied using NIST-SRM981 reference material. The Pb isotopic composition of procedural blanks did not influence significantly the $^{206}Pb/^{207}Pb$ and $^{206}Pb/^{208}Pb$ ratios measured in all samples. The coefficients of variation of the NIST-SRM981 reference material obtained in between-batch external quality control were 0.37 % for $^{206}Pb/^{207}Pb$ and 0.22 % for $^{206}Pb/^{208}Pb$ ratios.

Sub-cellular fractionation

Fresh samples were homogenised at a dilution of 1:3 (wet weight:volume of buffer) in an ice bucket. The buffer consisted of Tris-HCl (10 mM, pH 7.4, and 0.15M NaCl) and 1mM PMSF (phenylmethylsulfonylfluoride, as protease inhibitor). The homogenation was performed by hand and completed in approximately 5 min. to minimize organelle breakage.

Each homogenate was transferred to centrifuge tubes and subjected to differential fractionation. The procedure adapted from Campbell et al. (2005) is schematically the following: the homogenate was first fractioned by centrifugation at 700 x g for 15 min at 4ºC to separate the nucleus; the supernatant was further centrifuged at 9 000 x g for 20 min at 4ºC to separate the mitochondrial fraction; the lysosome and microsomal fractions were obtained by centrifuging the supernatant at 30 000 x g for 25 min, and at 100 000 x g for 40 min at 4ºC, respectively. The four fractions obtained by the centrifugation procedure were lyophilized for metal analyses.

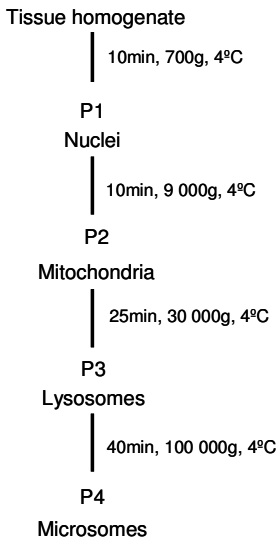

Figure Ap.2 – Schematic procedure of the sub-cellular fractionation by sequential centrifugation (adapted from Campbell et al., 2005).

The procedure adapted from Wallace et al. (2003) and Campbell et al. (2005) comprised five operationally defined fractions (Figure 3). The three “particulate” fractions are granules, mitochondrias, lysosomes and microsomes. The two “cytosolic” fractions consist of heat-stable proteins (HSP), including metallothioneins and glutathione, and heat-denaturable proteins (HDP) containing enzymes and other non-enzymatic proteins. The five fractions were obtained by the following centrifugation procedure: the aliquot was firstly centrifuged at 800g for 15 min at 4ºC (P1 and S1). The P1 that contained nuclei, unbroken cells, cell membranes and granules was re-suspended in initial buffer (1:3, m:v), heated at 100ºC for 2 min, 1N NaOH was added and heated again at 60-70ºC for 10min, after that a new centrifugation was made at 10 000 x g for 30 min at 20ºC. Two fractions were obtained, only the pellet (P2) with granules was further used. The supernatant S1, was centrifuged sequentially to separate P3 the mitochondria fraction, at 10 000 x g for 30 min at 4ºC, the lysosome and microsomal fractions (P4) were obtained by further centrifuging the supernatant at 100 000 x g, for 60 min at 4ºC. The “cytosolic” fractions (P5 and S5) were separated by heating the S4 at 80ºC for 10 min and centrifuging at 50 000 x g for 15 min at 4ºC. The heat-stable proteins (HSP) remain in the final supernatant. The five fractions obtained by the centrifugation procedure were lyophilized for trace element determination.

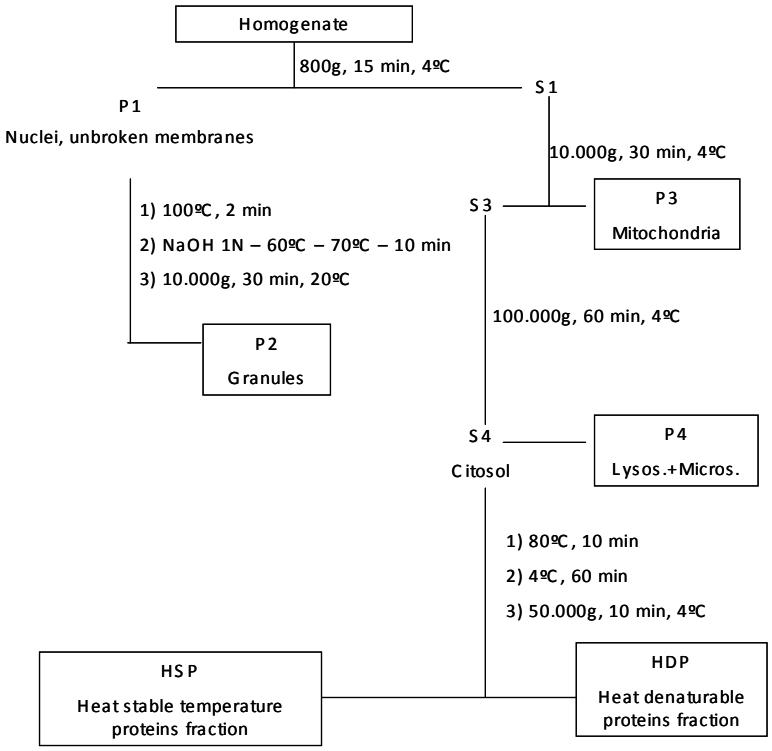

Figure Ap.3 – Schematic procedure of the sub-cellular fractionation by sequential centrifugation (adapted from Wallace et al., 2003 and Campbell et al., 2005).

Protein purification

Fresh samples were homogenised at a dilution of 1:3 (wet weight:volume of buffer) in an ice bucket. The buffer consisted of Tris-HCl (10 mM, pH 7.4, and 0.15M NaCl) and 1mM PMSF (phenylmethylsulfonylfluoride, as protease inhibitor). The homogenate was centrifuged at 100 000 x g for 1h at 4ºC and the supernatants were carefully pipetted off and immediately stored at -80ºC. The soluble clear solution was applied to a gel filtration column (Sephadex G-75, 2.6 x 89 cm) equilibrated with Tris-HCl (10mM, pH 7.4, 0.15M NaCl). The column temperature was maintained at 4ºC. The supernatants were applied to the column using volumes of 5mL. Elution was performed at a flow rate of 0.3 mL/min and fractions of approximately 4mL were collected. The column was calibrated with standards of the different molecular weight: blue dextran (approx. 2 000 000 Da), albumin (67 000 Da), ovalbumin (43 000 Da), chymotrypsinogen A (25 000 Da) and ribonuclease A (13 700 Da). Absorption at 254 and 280 nm as well as the concentrations of Zn, Cu, Cd and Pb were measured in each fraction.

Metallothionein analyses

Fresh samples were homogenised in cold (4 ºC) TRIS–HCl 0.02M buffer (pH 8.6) using a Potter–Elvehjem homogenizer, in an approximate proportion of 1:3 tissue ww:buffer volume. Homogenates were centrifuged at 30 000 × g (1h at 4 ºC) and the supernatant (cytosol) was heated at 80 ºC for 10 min to denaturate non–heat resistant proteins. Heat–treated cytosols were then centrifuged at 50 000 × g (30 min h at 4 ºC) to precipitate the non–heat resistant and remaining high molecular weight proteins. Metallothionein in heat–treated cytosols was determined by differential pulse polarography (DPP) with a static mercury drop electrode (SMDE) using a 694 stand and a 693 processor (Metrohm). The electrode system consisted in a mercury capillary working electrode, an Ag/AgCl reference electrode and a platinum auxiliary electrode. The supporting electrolyte (1M NH_4Cl, 1M NH_4OH and 2mM $[Co(NH_3)_6]Cl_3$) was prepared weekly and stored at 4 ºC as described by Palecek and Pechan (1971). In absence of a commercial mollusc MT, Rabbit MT (forms I & II, from Sigma) was used for quantification of thiols using a standard–addition technique. The marked similarity between the polarogrammes generated by rabitt and octopus MTs confirmed the suitability of using rabitt MT to calibrate the assay for octopus. The procedure followed Costa et al (2008a) methodology that was adapted from Bebianno and Langston (1989). Results are expressed as mg MT–equivalent g^{-1} tissue dry mass (dm).

DNA strand breakages

DNA total strand breakage (DNA-SB) was assessed through the alkaline single–cell gel electrophoresis (Comet) assay (Singh et al., 1988) and adapted from the method described by Costa et al. (2008). Aliquots of fresh digestive gland, gill, kidney and gonad cells were ressuspended in KSS (Kenny's Salt Solution) 1:1 (w/v), and placed on slides pre-coated with 1% (w/v) normal melting-point agarose in TAE buffer (the slides were allowed to dry for at least 48 h) and covered with a coverslip. After agarose solidification (15min, 4 ºC) the coverslip was removed and the slides were dipped for 1 h at 4 ºC in lysis solution (2.64% NaCl (w/v), 3.72% EDTA (w/v) and 5mM TRIS) to which was added 10% (v/v) DMSO and 1% (v/v) Triton-X 100 just before use. Slides were afterwards placed in cold (4 ºC) electrophoresis solution (pH 13) for 40 min to allow DNA-unwinding and enhanced expression of alkali-labile sites. Electrophoresis was for 30 min at 25 V, in the cold (4 ºC), using a Sub-Cell model 96 apparatus (Bio-Rad). Slides were afterwards neutralized in 0.1M Tris–HCl buffer (pH 7.5) for 15 min. All preparatory steps were performed under controlled temperature (≈16 ºC) to avoid gel lifting from the slides and all solutions and electrophoresis apparatus were kept in the dark and in the cold to minimize accessory DNA degradation. Approximately 100 comets were analysed per slide after staining with 0.02mg mL^{-1} ethidium bromide (EtBr). Comets were analysed using the software CometScore 1.5 (TriTek Corp., Summerduck, USA). The percentage DNA in the tail was employed as a direct measure of DNA-SB (Lee and Steinert, 2003). DMLB microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps was used, equipped with an N2.1 filter, all from Leica Microsystems.

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