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Assessment of metals contamination (Cd, Pb, Ni) at the Óbidos Lagoon using* Cerastoderma edule *as a biomonitoring tool

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Assessment of metals contamination (Cd, Pb, Ni) at the Óbidos Lagoon using **Cerastoderma edule** *as a biomonitoring tool*

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"O renovar é uma constante…

Por isso, segue as ondas do teu coração."

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RESUMO

Resumo

A poluição marinha é um problema ambiental Mundial que afeta as constituintes bióticas e abióticas do Ecossistema. Os resultados de diversas atividades antropogénicas chegam ao meio marinho através de escorrências terrestres, industriais e agrícolas assim como através de descargas domésticas, causando distúrbios neste Ecossistema. Sendo assim, a avaliação da contaminação por metais é uma das prioridades tendo em conta que estes químicos têm comportamentos acumulativos e podem ser transportados ao longo de grandes distâncias.

Nos estuários, os bivalves são componentes chaves das comunidades macrobentónicas devido o seu papel vital na comunidade e também por serem uma ótima fonte de alimentação para os seres Humanos. Na Lagoa de Óbidos, uma das espécies com estas características e intensamente apanhada é o berbigão *Cerastoderma edule*. Este berbigão foi descrito por vários autores como sendo tolerante a diversos poluentes. Sendo assim, no presente estudo, o principal objetivo foi utilizar *C. edule* como ferramenta de biomonitorização de contaminações por metais na Lagoa de Óbidos, avaliando a contaminação por Cádmio, Chumbo e Níquel durante as estações do ano de 2009 e 2010 em duas estações de amostragens (estação ML e BSB). Os resultados foram complementados com a análise de contaminações por metais em amostras de águas e sedimentos. Diversas respostas fisiológicas do berbigão foram também averiguadas de modo a perceber os efeitos da presença de metais e sua acumulação. Finalmente, avaliou-se a Dose Semanal Admissível Provisória (DSAP) para o consumo de berbigão para os mínimos e máximos de concentrações de metais detetados em cada estação de amostragem.

Todos os metais foram detetados em amostras de água enquanto apenas o Pb foi detetado no sedimento. O Pb e o Ni foram detetados mais vezes na amostras biológicas do que o Cd. No entanto, as contaminações por metais Pb e Cd no berbigão foram frequentemente acima do limiar fixado pelas autoridades responsáveis enquanto o Ni se encontrou perto dos valores legislados. A avaliação da DSAP revelou a necessidade de aumentar a monitorização biológica neste berbigão visto que a dose semanal admissível foi muito baixa para os três metais. De um modo geral, *C. edule* refletiu as modificações ambientais, respondendo a modificações físico-químicas e contaminações por metais durante o período de estudo em ambas as estações de amostragem. Assim, este estudo permitiu concluir que *Cerastoderma edule* foi um bom e sensível indicador de contaminações por metais, especialmente para o Pb e preferencialmente para o Ni.

Palavras-chave: Biomonitorização, Cádmio, Chumbo, Níquel, Lagoa de Óbidos, *Cerastoderma edule*

ABSTRACT

Abstract

Marine pollution is a World environmental problem and may affect both the biotic and abiotic components of the ecosystem. The results of many anthropogenic activities can arrive to the marine environment by terrestrial runoff, and industrial, agricultural and domestic discharges, causing disturbances on this system. The assessment of the metal contamination is one of the priorities since these chemicals have accumulative behaviours and have a long range transport.

In estuaries, bivalves are an important component of the macrobenthic communities, due to their vital role on the community energy flow, and are also a good resource for Human consumption. At the Óbidos Lagoon, one of the species with these characteristics, and harvested as shellfish, is the common cockle *Cerastoderma edule.* This cockle was reported as tolerant to pollutants by some authors. As such, on the present study, the main objective was to use *C. edule* as a biomonitoring tool for contamination by metals at the Óbidos Lagoon, by evaluating the contamination by Cadmium, Lead and Nickel on a seasonal basis, from 2009 to 2010, at two different stations (ML and BSB station). The results were complemented with the analysis of metal contamination on water and sediment samples. Some physiological responses of the cockles were also studied in an attempt to understand the effects of the metals presence and their accumulation. Finally, the Provisional Tolerable Weekly Intake (PTWI) for the consumption of cockles' for each minimal and maximum concentrations of metal detected at each station was evaluated.

All the metals were detected on water samples while only Pb was observed on sediments samples. Pb and Ni were detected most often on biological samples than Cd. However, Pb and Cd contamination on cockles were frequently above the threshold fixed by the legal authorities, while Ni contaminations were close to the values legislated. The PTWI assessment revealed the need to increase metal biomonitoring on this cockle since the weekly intake of this bivalve was very low for the three metals. At a global analysis, *C. edule* reflected the environmental changes, responding to physicochemical changes and metal contaminations along the study period and at both stations. So, this study allowed concluding that *Cerastoderma edule* was a good and sensitive indicator of metals contamination, especially for Pb and preferentially for Ni.

Key words: Biomonitoring, Cadmium, Lead, Nickel, Óbidos Lagoon, *Cerastoderma edule*

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LIST OF ABBREVIATIONS & ACRONYMS

List of Abbreviations & Acronyms

[Cd]	Cadmium concentration
[Ni]	Nickel concentration
[Pb]	Lead concentration
AAS	Atomic Absorption Spectrometry
BAF	Bioaccumulation factor
BAF_{max}	Maximum bioaccumulation factor
BAF_{min}	Minimum bioaccumulation factor
BSB	*Bom Sucesso* Branch
C. edule	Cockles *Cerastoderma edule*
Cd	Chemical element Cadmium
CF	Concentration factor
C_o	Concentration of the element in the soft tissue of the cockle
C_s	Concentration of the element in the sediment
C_{sw}	Concentration of the element in the seawater
Cu	Chemical element Copper
C_x	Concentration of the element in the soft tissue of the cockle
d.w.	Dry weight
df	Degrees of freedom
EEA	Environmental European Agency
EFSA	European Food Safe Authorities
ELV	Emission Limit Value
EQS-AM	Environmental Quality Standard-Arithmethic Mean
EU WFD	European Water Frame-work Directive
Fe	Chemical element Iron
FSANZ	Food Standards Australia and New Zealand
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
h	Hour
H	Height
Hg	Chemical element Mercury
HNO_3	Nitric acid
JECFA	Joint FAO/WHO Expert Committee on Food Additives
km^2	Kilometers per square
$l.h^{-1}.g$	Liters per hour per gram

$l.kg^{-1}$	Liters per kilogram
M	Meter(s)
$m^3.s^{-1}$	Cubic meters per second
mA	Milliamp
MAV	Maximum Admissible Value
$mg.kg^{-1}$	Milligrams per kilogram
$mg.l^{-1}$	Milligrams per liter
ml	Milliliter(s)
ML	Middle Lagoon
mm	Millimeter(s)
Mn	Chemical element Manganese
MS	Mean square
N	Sample number
Ni	Chemical element Nickel
NW-SE	Northwest-Southeast
Ø	Diameter
OM	Organic matter, %
Pb	Chemical element Lead
PET	Polyethylene terephthalate
PTWI	Provisional Tolerable Weekly Intake
S	Salinity
SE	Southeast
SNIRH	Sistema Nacional de Informação de Recursos Hídricos
SW	Southwest
T	Temperature
USFDA	United States Food and Drug Administration
W	West
w.w	Wet weight
Z	F-statistic
$\rho_{spearman}$	Spearman’s correlation

1. INTRODUCTION

1. Introduction

1.1. Marine environmental pollution

Marine pollution is a global environmental problem and may affect both the biotic and abiotic components of the ecosystem (Di Bella *et al.*, 2013; Pedro *et al.*, 2013). Different anthropogenic activities on land, water or air such as urban runoff, industrial and rural activities, agricultural effluents and domestic discharges, are contributing to the contamination of seawater, sediment and organisms with potential accumulation of toxic substances (Roberts *et al.*, 2008; Di Bella *et al.*, 2013; Pedro *et al.*, 2013).

The first areas exposed to the contamination with pollutants are the coastal and estuarine zones (Di Bella *et al.*, 2013; Pedro *et al.*, 2013). In the estuarine environment, after the pollutants are discharged, the contaminants can follow three ways. They can stay in the water in the dissolved form or they can be removed from the water column through sedimentation to the bottom sediments or they can even be taken up by the organisms (Zhao *et al.*, 2013).

In semi-enclosed systems such as a lagoon with narrow opening, a significant part of the pollutants remains closer to their land-based pollution sources due to their lower ability to flush and disperse the contaminants out to the open sea than coastal areas with open boundaries with the sea, often leaving a long term pollution legacy (Williams & Hudson, 2014).

In the recent years, Pedro *et al.* (2013), has studied the capability of *Salicornia ramosissima* J. Woods to bioaccumulate cadmium at different salinities, while Nilin *et al.*, (2012) has studied the physiological responses of *Cerastoderma edule* L. to pollution, by analysing responses such as oxygen consumption and clearance rate, survival in air, condition index and total energy content, and assessed their use as indicators of mercury pollution at *Ria de Aveiro*. Hussein & Khaled (2014) have studied the levels of some heavy metals in the muscle, liver and gill tissues of tuna fish as well as in bivalves from three hot spots along the Alexandria coastal water, and assess the public health risks associated with the consumption of mussels and the edible portion of the fish harvested. Therefore, the contamination with metals has been intensively studied because these pollutants are persistent, toxic, have an accumulative behaviour and potential for long-range transport (Di Bella *et al.*, 2013; Pedro *et al.*, 2013). These characteristics are a risk to Ecosystems and Human Health (neurological disease and cancers), so to understand this problem, studies are focused on estuarine and coastal environments (Fraser *et al.*, 2011).

1.2. Estuarine Habitats – the Óbidos Lagoon as a case study

The estuarine habitats are among the most productive environments of the coastal zone (Crespo *et al.*, 2010; Cardoso *et al.*, 2013). Thus, they are important areas with high economic value. The high economic value of these ecosystems is due to the fact that such areas are used as harbours and areas for food supply. Nowadays, many Human applications are developed in these aquatic systems, namely aquaculture and fisheries, industrial, recreational and leisure activities. It is relevant to refer that these systems are important nursery areas and feeding grounds for many itinerant marine animals and bird communities (Correia *et al.*, 2012)

Coastal lagoons are considered a type of estuarine habitats, formed by shallow water bodies partially isolated from the adjacent sea by a sedimentary barrier. These systems are considered different from “true” estuaries mainly on a geomorphologic and hydrographical origin, whereas from an ecological standpoint coastal lagoons and estuaries represent similar ecosystems. Marine and Terrestrial factors are influenced directly by the position between land and sea (Carvalho *et al.*, 2005). For these reasons, the Environmental European Agency (EEA) designates such zones as physically sensitive areas due to their intermittency of connection with the sea (Correia *et al.*, 2012)

In a typical lagoon, the exchange and mixture of saltwater and freshwater is irregular and the hydrography can have fluctuations daily, seasonally or at the long-term. Biological communities in coastal lagoons are largely influenced by restricted freshwater inputs and by mixing and exchange processes with marine areas. Other factors interfere too as geographical location, climatic regimes, surrounding environment, internal biogeochemical cycles and degree of communication with the sea (Carvalho *et al.*, 2011; Correia *et al.*, 2012).

Another characteristic of the coastal lagoons is the vulnerability to eutrophication (Carvalho *et al.*, 2011), due to the restricted exchange with the adjacent sea, and consequently the nutrients accumulation exported by the river basin (Correia *et al.*, 2012).

These small systems are easily disturbed by natural processes or by the increased nutrient inputs due to anthropogenic activities, such as modification of land use, effluent disposal and aquaculture or by pollution or yet, by adjacent urban and industrial development (Correia *et al.*, 2012; Carvalho *et al.*, 2005). The eutrophication can lead the system to low water oxygenation and thereby to the production of toxic hydrogen sulphide. In some cases, anoxic conditions can even occur in the sediment leading to metal release and nutrients to the overlying water, during the night. Thus, coastal lagoons may experience an additional problem of metal contamination, with potential repercussions in the biota (Carvalho *et al.*, 2011).

The Óbidos Lagoon, situated in the Western coast of Portugal between Cape *Carvoeiro* and *São Martinho do Porto*, is one of the largest coastal lagoons of the country. This lagoon ecosystem has a preferred orientation NW-SE, but this orientation can move depending on the tidal oscillation and the sand entrance to the natural system (ICN *et al.*, 2005).

This coastal system is situated in the municipalities of *Caldas da Rainha* and *Óbidos*, and is located in a shallow depression, has irregular contours and is very unstable due to the proximity of the sea. The natural barrier, that separates the lagoon from the marine environment, is formed by a string of coastal dunes. Their connection with the sea side is through a channel with a wide and variable positioning called "*A Aberta*". Sometimes this connection closes and human intervention is needed in order to maintain the open bar **(Figure 1.1;** ICN *et al.*, 2005).

Figure 1.1: Location of Óbidos Lagoon. Municipalities of *Caldas da Rainha* (top right) and *Óbidos* (bottom & left) (Governo de Portugal - Secretário de Estado da Cultura, 2012).

The Óbidos Lagoon comprises extensive intertidal sandbanks partially separated by more or less deep channels, and extends upstream by two channels, to the East by *Barrosa*'s Arm and to the SW by the *Bom Sucesso* Arm which suffer small freshwater contributions (Carvalho *et al.*, 2005; ICN, 2005).

This lagoon system is recognized as an important ecological and socio-economical system. Ecologically, it houses a large number of different and complex ecological niches (fauna, flora, landscape, ecosystems). Regarding the socio-economical relevance, several activities depend of the Óbidos Lagoon, namely shellfish harvesting, fisheries (activities with greater social and economic importance), tourism, agriculture, livestock, industry, commerce and services (ICN, 2005).

According to Carvalho *et al.* (2011), the Óbidos Lagoon is moderately contaminated by metals and has experienced high nutrient loads of anthropogenic origin (particularly nitrogen and phosphorus) leading to signs of eutrophication. In coastal lagoons, this is a common environmental problem that can result in community dominance changes, as well as modifications in species composition (Carvalho *et al.*, 2011).

1.3. Environmental biotechnology as a monitoring and assessment tool for metals

Metals and others elements are naturally released in to the marine environment through weathering and leaching processes (Figueira *et al.*, 2011), since they are natural components of the Earth's crust. However, and mainly in the last decades, metals have also been discharged through industrial and domestic sewage discharges, mining, smelting, and e-wastes recycling (Zhao *et al.*, 2013), increasing the contamination of the marine environment specially on estuarine and coastal waters. Therefore, this environmental situation is a real problem that concerns the Whole World (Figueira *et al.*, 2011; Williams & Hudson, 2014; Zhao *et al.*, 2013).

The biggest environmental problem with metals is the accumulation behaviour and their toxicity, as well as their potential for long-range transport depending on the system. Another problem is the capacity of metals to remain in solution in the water column or in suspension and becoming available to precipitate onto the bottom sediments or be taken up by organisms through adsorption, respiration, and ingestion. All of these behaviours may affect the delicate ecological balance of natural systems and specially the organisms living there, as well as high trophic levels namely Humans through the consumption of seafood (Zhao *et al.*, 2013; Williams & Hudson, 2014).

To assess the environmental quality of coastal ecosystems different environmental indicators, such as surface water and sediments chemical analyses, may be used. In the last years, however, the need to study biological samples to assess the metal bioaccumulation on aquatic organisms arose, and biomonitoring of the coastal environment emerged as an environmental quality assessment tool (Pereira *et al*, 2012). Some species of invertebrates are known as efficient accumulators of metals, for example mussels (*Mytilus edulis* L.; Fraser *et al.*, 2011), Manila clams (*Ruditapes philippinarum* Adams & Reeve, 1850; Zhao *et al.*, 2013), as well as macrophytes (e. g. *Bolboschoenus maritimus* L., Santos *et al.*, 2015). This method provides valuable information on the temporal and spatial variations of bioavailable metal contaminants within the environment (Williams & Hudson, 2014). According to the Directive 2000/60/CE of the European Parliament and of the Council, "Environmental Quality Standard" is the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment (Directive 2000/60/CE). The line between essential and harmful quantity of metal is usually complex and the metal contamination in water and food are difficult to establish (Zhao *et al.*, 2013).

The level of metal bioaccumulation by aquatic organisms depends on several environmental variables, such as temperature, salinity, seasonal variations and on various biological variables such as diet, spawning, organisms and obviously on their chemical characteristics such as essentiality and toxicity. The accumulated metals can be classified in two types: 1) metal that has been detoxified or 2) metal that is metabolically obtainable to satisfy essential requirements or to interact in a fashion that expresses itself as a toxic response (Conti *et al.*, 2011; Zhao *et al.*, 2013; Williams & Hudson, 2014).

The Directive 2008/105/CE of the European Parliament and of the Council, December 16th and consequently the Portuguese Legislation, Decree-Law n°103/2010, September 24th, defines a list of priority substances in the field of water policy. According to these documents, Cadmium (Cd) is classified as a priority hazardous substance (substances or groups of toxic, persistent and liable to bioaccumulate, and other substances of concern in the same order), while Lead (Pb) is classified as a dangerous metal that can be very harmful even at trace levels during prolonged exposure. Finally, Nickel (Ni) is just classified as a priority substance (Zhao *et al.*, 2013).

1.3.1. Cadmium (Cd)

Cadmium (Cd) element can be found mainly in the earth's crust and occurs always in combination with zinc, while the metallic form is rarely found in the environment, occurring in the salt form. It is released to the environment by means of natural (from weathering of rocks) or by air (through forest fires and volcanoes) and/or anthropogenic ways (WHO, 2011). This element is non-essential and acknowledged as an extremely hazardous pollutant due to its large solubility in water and high toxicity, and biopersistent to most organisms (Pedro *et al.*, 2013) until being excreted. Some cadmium salts, such as the sulphide, carbonate and oxide are particularly insoluble in water; these forms can be converted to water-soluble salts in nature (OSPAR, 2004).

The main anthropogenic application of Cd is in Ni / Cd batteries. Other uses of this element are as coatings, with good anti-corrosive properties (for example: marine & aerospace applications), or as pigments, plating, stabilizers for plastics, alloys and electronic compounds, but also as compounds of manures and pesticides (OSPAR, 2004). Cd can be obtained too as a sub-product for example on production of phosphate fertilizers, detergents, refined petroleum products and zinc, lead and copper extraction (OSPAR, 2004; Alfonso *et al.*, 2008).

Various soil organisms, such as earthworms are extremely susceptive to Cd poisoning and can die at very low concentrations. In marine ecosystems, Cd can bioaccumulate in mussels, oysters, shrimps, lobsters and fish. For example, Neff (2002) has reported that crabs and lobsters are able to accumulate, between 10 and 40 % of the cadmium present in their food (EFSA, 2009). It was even reported that some fishes (*Solea lascaris* Risso*, Lophius budegassal* Spinola and *Triglia lucerna* L.) can accumulate this non-essential metal in their tissues (Yilmaz *et al.*, 2010).

In Humans, the major exposure pathway is through food and after by air and drinking water. Harmfulness is associated with renal dysfunction (WHO, 2011) and obstructive lung disease, but it may also produce bone defects (osteomalacia, osteoporosis). Cd can be linked to increased blood pressure, liver disease and nerve or brain damage (OSPAR, 2004).

For Cd and their compounds, depending on the water hardness, Decree-Law n°103/2010 has defined the Environmental Quality Standard-Arithmethic Mean (EQS-AM) values in surface waters between $\leq 0.08 \times 10^{-3}$ and 0.25×10^{-3} mg.l^{-1}.

1.3.2. Lead (Pb)

Lead (Pb) element arises in the ecosystem by natural (volcanic activity, glacier movements, floods, forest fires, meteorites, erosion) and anthropogenic sources (WHO, 2011). Its median content in worldwide ocean waters is in trace amounts between 0.03ug.l^{-1} and 0.003ug.l^{-1} but in rivers the concentration is highest, 0.08ug.l^{-1} (EFSA, 2010).

This element is non-essential, acknowledged as a toxic element even in trace amounts (Fatoki *et al.*, 2012), persistent and cannot be degraded in to harmless substances (OSPAR, 2003). The element Pb has various applications such as in manufacturing, construction and chemical industries being found in lead-acid batteries, petrol additives, paint production, roofs and stained glass windows, rolled and extruded products, alloys, pigments and compounds, solder (in water pipes), fertilizers and ammunition industry (WHO, 2011).

Pb is insoluble in water under standard conditions (T=20^{o}C & P= 1 bar). However, in water it appears in ionic form (Pb^{2+}), with high mobility and bioavailability or in organic complexes with dissolved humic materials or attached to solid particles of clay. It is known that Pb is generally soluble in soft, slightly acidic water (EFSA, 2010).

Ecotoxicologically, Pb-salts and organic Pb compounds are the most harmful and toxic pollutants. Exposure to this metal can occur through air, food, drinking water, soil and dust from old paint with Pb (EFSA, 2010).

Dissolved or suspended forms of Pb have origins in wastewater that stems from streets, pipes and soils. Due to all of these characteristics, Pb is easily accumulated in organisms, sediments and sludge and may be easily transferred to higher trophic levels through the food chains. Bourgoin *et al.* (1990) reported high lead concentration in mussel near the Belledune. Burger *et al.*, (2002) reported that lead can cause deficits or decreases in the survival, growth rates, development and metabolism of fish.

Lead is well-known as a harmful substance and can accumulate in the body (in skeleton) and affect some organ systems such as the blood, the renal, endocrine, gastrointestinal, immune and cardiovascular system and the male and female reproductive organs. The most critical target appears to be the central and peripheral nervous systems, especially the brain of the fetus and infant (Landrigan *et al.*, 2000; EFSA, 2010).

For Pb and their compounds, Decree-Law n°103/2010 set a value of EQS-AM at 7.2×10^{-3} mg.l^{-1} in surface water.

1.3.3. Nickel (Ni)

Nickel (Ni) is one of the most abundant elements in the earth's crust (Coman *et al.*, 2013). In the marine environment it can have different origins (air, terrestrial, anthropogenic sources) and occur in a range from 1.0×10^{-4} mg.l^{-1} and 5.0×10^{-4} mg.l^{-1}. This essential element is a part of various biological cycles and can be found accumulated in sediments, slate, sandstone, clay minerals and basalt (Von Burg, 1997).

In industries, this element is used in many applications such as Ni-Cd batteries, as protective coating on steel and copper objects, as alloys (Ni-copper in coins, treatment of others metal polluted surface water or applied for kitchen ware, jewelry and turbine production), as anti-corrosive components, as a mordant in textile printing (Ni acetate), as catalysers or pigments (Ni carbonate and Ni chloride), as cleaning products (Nitetra-carbonyl), or as fertilizers in agriculture (WHO, 2011).

Ni is water insoluble at standard conditions ($T = 20^oC$ & $P = 1$ bar). Thus, some Ni compounds may be water soluble, namely Ni chloride, the most water soluble (553 g.l^{-1} at 20^oC to 880 g.l^{-1} at 99.9^oC) or Ni carbonate (90 mg.l^{-1}), whereas other Ni compounds, such as Ni oxide, Ni sulphide and Ni tetra-carbonyl are water insoluble (Von Burg, 1997).

In many organisms, this metal is a dietary requirement, but in larger doses it may be toxic. For example, in algae, nickel causes growth restraints at concentrations between 0.5 and 10 mg.l^{-1}, in *Daphnia hyaline* Müller the LD_{50} for 96 hours was 0.5 mg.l^{-1}, in freshwater bivalve is 1100 mg.l^{-1} and for marine lobsters the LD_{50} was higher 150-300 mg.l^{-1}, (Von Burg, 1997)

In the Human body, Ni is needed to produce red blood cells and probably in urea to ammonia conversion by the urease enzyme. Though, high exposure can cause different pathological effect as a contact dermatitis, a lung fibrosis (Coman *et al.*, 2013), a cardiovascular and kidney disease and even teratogenic and carcinogenic effects (WHO, 2011).Decree-Law n°103/2010 has defined 20×10^{-3} mg.l^{-1} in surface water as a value of EQS-AM for Ni and their compounds.

Decree-Law n°103/2010 has defined 20×10^{-3} $mg.l^{-1}$ in surface water as a value of EQS-AM for Ni and their compounds.

1.4. The importance of cockles as biomonitors of contaminants

To assess and monitor metal contamination in the aquatic ecosystems many species that belong at biogenic habitats are exploited as biomonitors because of their clear ecological importance. There are several examples of organisms used as biomonitors of metal availability in the marine literature, like for instance macroalgae, seagrasses, sponges and bivalve molluscs (Usero *et al.*, 2005; Roberts *et al.*, 2008).

The benthic macroinvertebrates play an important role in coastal ecosystems, due to their vital role in detritus decomposition, nutrient cycling and energy flow to higher trophic levels. And still, according to the Water Frame-work Directive (EU WFD - (WFD, 2000/60/EC)), benthic communities are one of the biological elements to assess ecological quality status to complete the traditional chemical analyses (Roberts *et al.*, 2008; Carvalho *et al.*, 2011; Correia *et al.*, 2012; Nilin et al., 2012; Di Bella *et al.*, 2013). An advantage of this approach is that the contaminant persists within the tissues for long periods of time (Roberts *et al.*, 2008; Conti *et al.*, 2011).

As referred before, bivalve molluscs are among the most used organisms as sensitive biomonitors for metal pollution in biological monitoring programmes in the marine environment, due to their characteristics as filter feeders (Conti *et al.*, 2011; Nilin *et al.*, 2012), fast response to environmental changes (Lobo *et al.*, 2010) and their capacity to bioaccumulate different elements from their food, seawater and proximate sediments (Fatoki *et al.*, 2012). Other attributes in favour as an excellent indicator are appropriate dimensions, sedentary lifestyle, and easy identification, handling and collection of organisms. Also, their ability to accumulate metal, without suffering mortality (Boening, 1999), in general, respects the levels found in their environment (Fatoki *et al.*, 2012; Zhao *et al.*, 2013) and, finally, their wide distribution in estuarine and coastal waters (Fatoki *et al.*, 2012; Zhao *et al.*, 2013). This set of characteristics are important because bivalve molluscs are sensitive to pollutants of an area and they may contribute to the transfer of metals to higher trophic levels (Zhao *et al.*, 2013) namely in the diet of mammals, particularly Humans (Conti *et al.*, 2011).

The European cockle *Cerastoderma edule* (Linnaeus, 1758) (**Figure 1.2**) are found along the European Atlantic coast from the Western Barents Sea and Northern Norway to the Iberian Peninsula, and also along the coast of West Africa to Senegal (Crespo *et al.*, 2010; Nilin *et al.*, 2012; FAO, 2015).

This cockle lives buried just under the bottom surface on sand, sandy mud, fine gravel bottoms or in the intertidal zone until a few meters deep (Dabouineau & Ponsero, 2011; Nilin *et al.*, 2012) frequently exhibiting high population density in marine, estuarine environments (Lobo *et al.*, 2010) and sandy bays with some arrival of freshwater. Population density can vary from a few individuals per square meter to thousands (Dabouineau & Ponsero, 2011; FAO, 2015).

The cockle is an active suspension-feeding bivalve, filtering water by their inhalant and exhalant siphons positioned on the surface of the sediment (Dabouineau & Ponsero, 2011; Nilin *et al.*, 2012). Water is filtered through the gills to keep phytoplankton, zooplankton and organic particulate matter adherent (Crespo *et al.*, 2010; Dabouineau & Ponsero, 2011).

*Cerastoderma edule m*ay live exceptionally until nine to ten years old but the normal average is 2 to 4 years (Dabouineau & Ponsero, 2011). They are gonochorics (distinct sexes with 40% males to 60% females) and their sexual maturity is generally reached during the second summer month (size between 15 and 20 mm, 15 to 18 months), although they may mature in their first year depending on their size more than the individual age (Dabouineau & Ponsero, 2011; MarLIN, 2006). Their reproduction can occur during all the year depending on the surrounding environment (Temperature). The

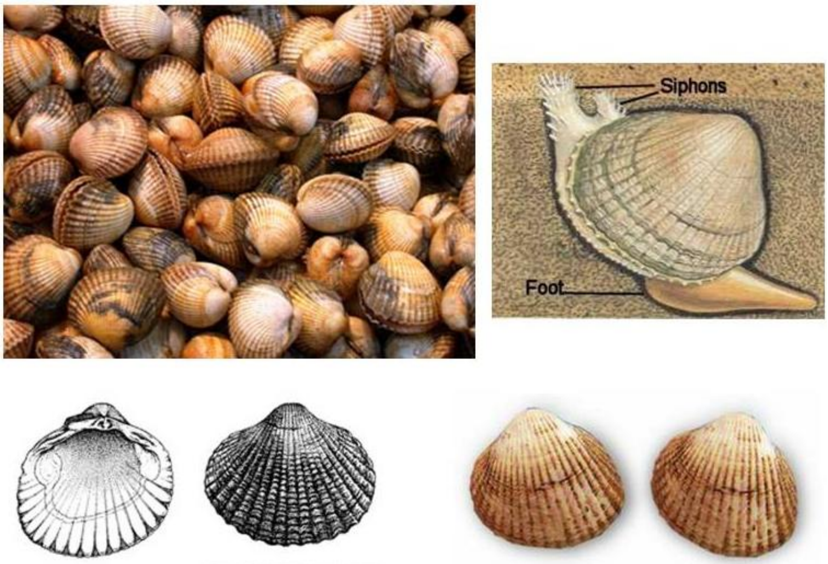


Figure 1.2: *Cerastoderma edule* (Linnaeus, 1758).

period of sexual dormancy corresponds to the coldest part of the year (Barnabé, 1994), normally this period correspond with the winter on the Óbidos Lagoon. Cockles grow rapidly in their first years (1-2 years) and then decline the growth rate (Dabouineau & Ponsero, 2011; MarLIN, 2006), while on winter months this species presents a low or negligible growth (Crespo *et al.*, 2010). They can reach a maximum length of 5.6 cm but the common length is between 3 and 4 cm. (FAO, 2015).

The mortality by natural and anthropogenic causes of this species is very high, oscillating between 50 to 70% of the population of the previous year (Dabouineau & Ponsero, 2011). The natural predation is higher on the first stages of growth and then it decreases. The crab *Carcinus maenas* L., the shrimps *Crangon crangon* L., the fish *Pomatoschistus microps* Krøyer, the polychaete worm *Hediste diversicolor* O.F. Müller and some birds have been reported as important predators of *C. edule* (Dabouineau & Ponsero, 2011; Crespo *et al.*, 2010) but the pressure by human’s predation is higher (Crespo *et al.*, 2010).

In Portugal, cockles are collected and marketed throughout the year and have the legal capture size allowed above 2.5cm. The main market is in Spain and of the 3.881t registered in Portugal commercial ports, only 300t are produced by aquaculture in marine or brackish water (Nilin *et al.*, 2012).

In 2007, the “*Direcção-geral das pescas e aquicultura”* reported that on the central region of Portugal, where the Óbidos Lagoon is located, an average of 1232.8 ton of cockles were landed (DGPA, 2007). On the Óbidos Lagoon, *C. edule* is one of the three bivalve species more harvested (*Ruditapes decussates* L.*; Venerupis pullastra* Montagu *and Venerupis aurea* Gmelin; ICN *et al.*, 2005). Consequently, this species is incorporated into the Human diet and for that reason has high commercial interest.

Like other bivalves and due to their tolerability to pollutants (Carvalho *et al.*, 2006) and tolerability to environmental variations of physicochemical parameters such as sediment grain size and salinity (Lobo *et al.*, 2010), *C. edule* was already used in previous studies as a bioindicator for assessment of environmental quality (Cheggour *et al.*, 2001; Figueira *et al.*, 2011).

1.5. Aims of the study

In Portugal, as in the other countries, the cockle *C. edule* is well distributed and plays an important role in some areas. Its major role is in trophic chains as a relevant source of food for crustaceans, fishes, some birds and for Human consumption so it has an important impact, both ecologically and economically.

The selection of this species in the present study related with the facts that this cockle is well established at the Óbidos Lagoon and it is a popular food in this area. On the other hand, it is known that the Óbidos Lagoon is moderately contaminated by metals (*e.g.* as reported by Pereira *et al*, 2012).

Therefore, and since this work is part of a larger environmental quality assessment program at this coastal ecosystem, the present work pretends to evaluate the metal contamination (Cd, Pb, Ni) at the Óbidos Lagoon, on a seasonal basis, using *C. edule* as a biomonitoring tool, during the years 2009 and 2010.

To accomplish this aim, the following specific objectives are proposed:

- Quantification of metals contamination (Cd, Pb, Ni) in environmental samples of water and sediments, collected at the Óbidos Lagoon.
- Quantification of the contamination by metals (Cd, Pb, Ni) in biological samples, of the commercial species *C. edule*, collected in the Óbidos Lagoon.
- Assessment of the effects of these contaminants on the physiological responses of *C. edule*, namely on their bioaccumulation capacity.
- Inference of the weekly intake of metals (Cd, Pb, Ni) through the consumption of this cockle in Human diet.

2. MATERIALS AND METHODS

2. Materials and Methods

2.1. Study area

The **Óbidos Lagoon** is a small and shallow coastal ecosystem, located on the West Central Coast of Portugal (39º24'N, 9º17'W) (Malhadas *et al.*, 2009a, b). This system has a hydrographic basin area of 440km^2 with a surrounding wet area of 6.9km^2, and a maximum length of 6km and width between 1 and 1.5km. The medium depth is of 3m and the maximum depth of 4m (ICN *et al.*, 2005). The Óbidos Lagoon is connected to the Atlantic Ocean through a narrow inlet with a sandy barrier (± 1.5km in length). This barrier is permanently maintained open to ensure water and sediment exchanges between the ocean and the lagoon (ICN *et al.*, 2005; Ferreira *et al.*, 2009).

The shallow lagoon is characterized by semi-diurnal tides with a range of 0.5 to 4.0 m, depending upon location and tidal phase (Malhadas *et al.*, 2009a). The tides induce strong tidal velocities and shallow channels (Bruneau *et al.*, 2011). Their influence extends across to the entire lagoon without pronounced longitudinal variation of salinity or stratification (Carvalho *et al.*, 2006; Malhadas *et al.*, 2009a, b).

The Óbidos Lagoon is characterized by two distinct regions, with different morphological hydrodynamic and sedimentary characteristics: the lower lagoon and the upper lagoon (Malhadas *et al.*, 2009b).

Lower lagoon is the part of the lagoon connected with the Atlantic Ocean and is characterised by several sand banks (0.25-2mm Ø grain size) and narrow channels with strong velocities (1.6 m.s^{-1} in the inlet and 1m.s^{-1} in the channels) and low residence time (less than 3 days) (Gordo & Cabral, 2001; Carvalho *et al.*, 2006; Malhadas *et al.*,2009a). Also, it has a high water renewal leading to the improvement of water quality in comparison with the other areas (Carvalho *et al.*, 2011).

Upper lagoon comprises the central area of the lagoon, two elongated arms (*Barrosa* branch and *Bom Sucesso* branch) and a small embayment in the South (*Poça das Ferrarias*; Fortunato & Oliveira, 2007). The central area of the lagoon has sandy-mud bottom sediments (0.06 – 0.25mm Ø grain size) and the arm's area are characterized by muddy bottom sediments (<0.06mm Ø grain size), with low velocities (0.4ms^{-1}) and high residence time (on the order of 3 weeks), and a small freshwater influence mainly in the winter by the *Arnóia/ Real* river (Gordo & Cabral, 2001; Carvalho *et al.*, 2006; Malhadas *et* *al.*, 2009a). This discharge represents 90% of the freshwater entering into the lagoon and is the major source of sediments, whose deposition has created an extensive sand bank (Malhadas *et al.*, 2009a). Comparing the global water input in the lagoon, the freshwater input plays a minor role, amounting annually to average flow rates in the order of $3m^3.s^{-1}$, which is less than 5% of the average tidal prism (Oliveira *et al.*, 2006; Pereira *et al.*, 2009b).

***Barrosa* branch** (SE) is a shallow area, with a mean depth between 0.5 & 1.0m. Most of the water circulation is due to the tides and to the small contribute of the Cal River ($0.14m^3s^{-1}$ average flow) (Carvalho *et al.*, 2011) which was classified as presenting deteriorated quality conditions according to the Portuguese classification of freshwater systems (Pereira *et al.*, 2009b; Micaelo *et al.*, 2010). This input is responsible for the entrance of urban effluents from the cities nearby, like *Caldas da Rainha* town (50.000 inhabitants) and some other pollutants, mostly from agricultural fields or livestock (Kowalski, 2009; Carvalho *et al.*, 2011). Consequently, this area presents the highest nutrient availability of the lagoon, being classified as eutrophic (Pereira *et al.*, 2009b).

***Bom Sucesso* branch** is a confined area too, with a mean depth range of 2-3m. The smaller freshwater uptake is received by *Ditch of Ameal* ($0.08m^3s^{-1}$ average flow) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems (Carvalho *et al.*, 2011).

Since *C. edule* is not present throughout all the margins of the lagoon, the selection of the sampling sites was restricted to two sampling areas, where *C. edule* was regularly present and in almost all seasons of the study period. One located at the Middle Lagoon (ML) in the right margin of the lagoon and another at the *Bom Sucesso* Branch of the lagoon (BSB) in the left margin of the lagoon (**Figure 2.1**).

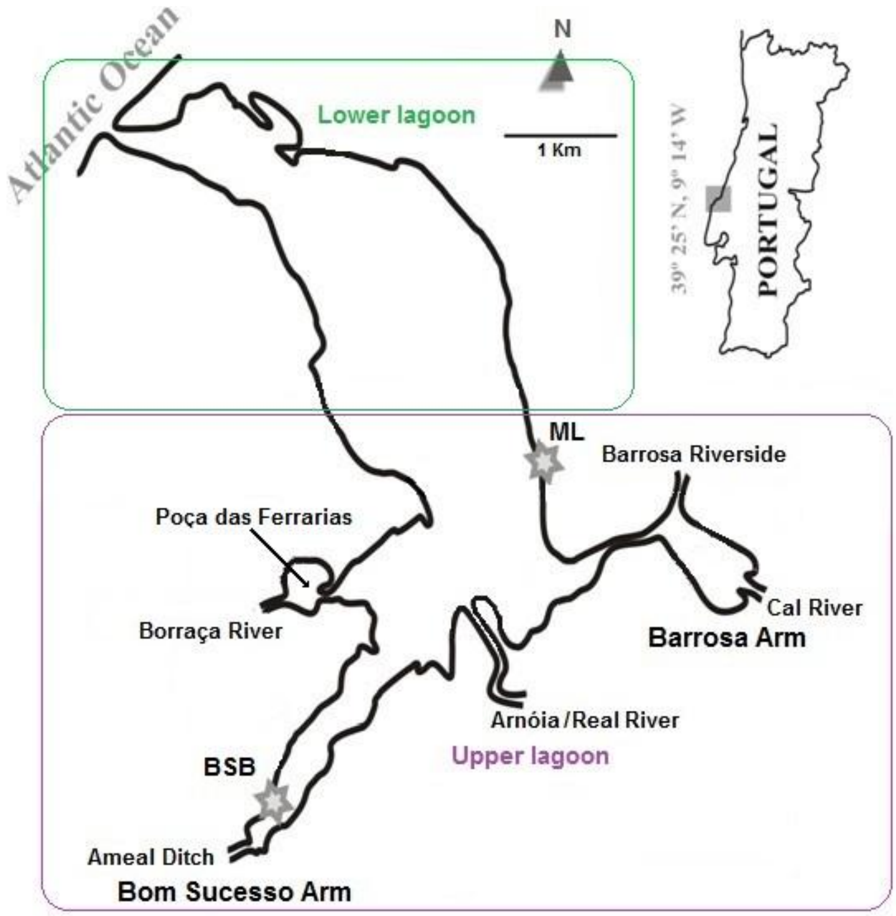


Figure 2.1: Location of the sampling sites in the Óbidos Lagoon. ML – Middle Lagoon; BSB – *Bom Sucesso* Branch

2.2. Sampling programme and procedure

Surface water, sediments and cockles were seasonally collected at low tide of spring tides, in intertidal areas submerged or recently exposed to air, at two sampling stations in the Óbidos Lagoon from February (winter) 2009 to July (summer) 2010. One of the sampling stations was located in the Middle Lagoon – ML – while the other one was located in the *Bom Sucesso* Branch – BSB – of the lagoon (**Figure 2.1**).

Monthly precipitation (mm) during the 7 periods of the study was obtained from *Sistema Nacional de Informação de Recursos Hídricos* (SNIRH). Water values of temperature (°C), salinity (‰) and pH were registered *in situ* using a portable multiparameter probe HANNA Hi 9828 (Woonsockets, EUA).

At each sampling station and at each sampling moment, one sample of surface water at < 0.5m depth was sampled to an inert plastic bottle (PET – polyethylene terephthalate), with

1.5L capacity. Also, one sample of the surface sediments was collected by hand, to approximately 5 cm below the surface, and stored in a plastic bag for later organic matter content (%) determination and metal analysis. All the samples were maintained in the dark, stored in a thermal container and transported to the laboratory for further treatment (USA EPA, 2000).

Regarding the biological samples, *C. edule* specimens were collected from the sediment by hand in the intertidal zones of each sampling station. Their valves were cleaned *in situ* to remove sediments, stored in black plastic bags, to avoid the light exposure, stored in a thermal container and transported to the laboratory, within 2h of collection.

2.3. Samples treatment

In order to avoid the contamination of the samples with metals, all the materials (glass and plastic) used in the field and in the laboratory, were washed by acidic wash. The acidic wash consisted in the immersion of the materials in 3% Derquim LM 02 (Panreac Química SAU, Spain) for 24h, washing with distilled water, and followed by immersion in 25% nitric acid (HNO_3 69%, PA-ACS-ISO, Panreac Química SAU, Spain) for 24h. After that, all the material was washed with ultra-pure water and dried in a laboratory oven at 60°C, following the procedure described in Pedro *et al.* (2013).

2.3.1. Lagoon water (dissolved & suspended fractions)

At the laboratory, 0.5l of the water samples were filtered per vacuum with a cellulose acetate membrane Whatman OE 67 (pore 0.45µm – 47mm Ø, Whatman GmbH, Dassel, Germany). The water previously filtered was afterwards acidified to $pH < 2$ with concentrated nitric acid (HNO_3 69%) (Scharlab,S.L., Barcelona, Espanha) and then transferred to 50ml in to plastic containers and frozen at -18°C until the atomic absorption spectrometry with graphite furnace atomization ((GFAAS) Thermo Scientific ICE 3500, Thermo Unicam, Portugal & SOLLAR FS95 Furnace autosampler) analyses to determine the presence of dissolved metals (Cardoso *et al.*, 2013; Zhao *et al.*, 2013).

The membranes used on the water filtration process were folded in 4 parts, conserving the suspended matter inside the membrane. After their duly identifications, the folded membranes were conserved in plastic bags with zipper in the freezer at -18°C until the determination of suspended metals (Fraser *et al.*, 2011; Pedro, 2011; Cardoso *et al.*, 2013).

Before the suspended metal analysis by GFAAS, the membranes were digested by acid digestion. The first step was to defreeze the membranes and dry them for 48h on a laboratory oven at 60°C. The dry weight was determined and registered. The acid digestion consisted in the complete dissolution of the membrane with two times addition of 3.0ml of 69% concentrated nitric acid at 100°C on a hot plate (VHP series C-10, VWR International - Material de Laboratório, Lda., Portugal). The temperature was increased at 200°C maximum until all the material was digested (a clear solution was obtained). The solution was evaporated to dryness (±1ml solution remained). After cooling, the residue was diluted with 3 ml of 1% HNO_3. The samples were filtered through Watman n° 41 filter paper (0.45µm and 55mm Ø; Whatman GmbH, Dassel, Germany) to collected the filtrate and discharge the suspended particles. Thus, the samples were diluted to a final volume of 50ml with ultra-pure water. Finally, until the GFAAS analysis, samples were frozen at -18°C (Hseu, 2004; Soylak & Cay, 2007; Pedro, 2011).

2.3.2. Surface sediments

At the laboratory, the sediments of each station and seasons of the study were dried at 60°C in a laboratory oven Memmert Drying UFB 500 (Memmert, Germany), during at least 48h, and stored on Deltalab hermetic weak 250ml (Deltalab S.L.U., Spain) until the subsequent study.

To determinate the content of organic matter (OM, %) of the lagoon substratum, the sediment was distributed on tree crucibles and dried in an oven lab at 60ºC, during 48h to determinate the dry weight. Then, the samples were burned at 500ºC, during 3h in a muffle, process was called loss weight on ignition (LOI). This last process allows obtaining the ash weight (Monterroso *et al.*, 2003). The OM was determined with the following equation:

$$\mathrm{O.M.}(\%) = \frac{\text{Dry weight} - \text{Ash weight}}{\text{Dry weight}} \text{ x } 100$$

Before the metal analysis by GFAAS, the surface sediments were crushed to a fine powder using a glass mortar. Approximately 100 mg of the crushed sediment were weighted and transferred to an Erlenmeyer of 50ml. Samples were digested by the acid digestion method. From this step on, the procedure was the same as described for the membrane nitric digestion (2.3.1).

2.3.3. Cockles

At the laboratory, the cockles were carefully placed in aquariums with continuous aeration and artificial marine water (S = 30‰; pH 8.0; T = 20 ± 2°C), renewed every day, to debug during 3 days. The main purpose of this procedure was to restart the respiration and filtration, and also purge the sediment inside the animal that could interfere with experimental procedures. After their duly identifications (station and sample season), the cockles were conserved in plastic bags in the freezer at -18°C until further treatment.

Before submitting the animals to the digestion process necessary for the further metal analysis, several biometric determinations were performed, as described below on section **2.5.1.1**. Afterwards, three animals of each sampling moment (replicates) were selected and submitted individually to a similar digestion process, as described before for the cellulose acetate membrane samples. However, only the soft part of the cockle and the intravalvular liquid were acidic digested, while the valves were discharged. **Figure 2.2** –

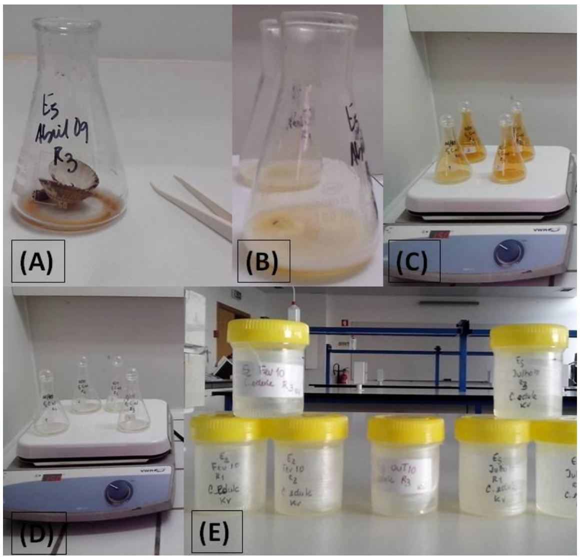


Figure 2.2: Acidic digestion on *C. edule* samples. (A) *C. edule* dried, after 48h at 60ºC; (B) Beginning of acidic digestion with HNO_3 69%; (C) Reactions occurring; (D) Complete digestion of the entire organism; (E) Final product used on GFAAS.

shows some steps of the acidic digestion process for *C. edule* samples.

2.4. Metals determination

Metals concentrations of Cd, Pb and Ni in the samples of water, membranes, sediments and cockles were determined using GFAAS analysis (**Figure 2.3**) using Argon (Air, Praxair Portugal Gases S.A., Portugal). The membranes, sediments and cockles' samples were digested according to the method described in the section **2.3.1**. Instrumental parameters and graphite furnace programs were those provided by the manufacturer.

Metal concentrations for water and membranes were determined as milligrams per liter, while for sediments and cockles the concentrations were determined on a dry weight basis as milligrams per kilogram. For water, membranes and sediments, metal analyses were performed using one replicate for each sampling moment. For cockles, the determination was performed with 3 replicates. All the standard solutions were daily prepared with ultra-pure water for metal analysis, from stock solutions.

Standard metal solutions (Cadmium standard solution, traceable to SRM from NIST $Cd(NO_3)_2$ in HNO_3 0.5 mol.l^{-1} 1000 ± 2 mg.l^{-1} Cd CertiPUR®, ©Merck KGaA, Darmstadt, Germany; Lead standard solution, traceable to SRM from NIST $Pb(NO_3)_2$ in HNO_3 0.5 mol.l^{-1} 999 ± 2 mg.l^{-1} Cd CertiPUR®, ©Merck KGaA, Darmstadt, Germany and Nickel standard solution, traceable to SRM from NIST $Ni(NO_3)_2$ in HNO_3 0.5 mol.l^{-1} 1000 ± 2 mg.l^{-1} Cd CertiPUR®, ©Merck KGaA, Darmstadt, Germany) were daily prepared for

Figure 2.3: Atomic absorption spectrometry with graphite furnace atomization (GFAAS) equipment used on the metal determination.

metal analysis, using a metal stock solution. For each sample aliquot, 20 ml of magnesium nitrate (Magnesium matrix modifier, for graphite furnace AAS c(Mg) = 10.0 ± 200 mg.l^{-1} ($Mg(NO_3)_2 + 6H_2O$ in HNO_3 ca. 17%, ©Merck KGaA, Darmstadt, Germany) as a chemical modifier was added, except for Nickel.

For each metal, the corresponding hollow cathode lamp (10 mA, Thermo Electron Corporation) was used. The detection limits of the AAS technique for each metal were the following: 0.00001 mg.l^{-1} for Cd; 0.0005 mg.l^{-1} for Ni; and 0.001 mg.l^{-1} for Pb (WHO, 2011).

The metal concentrations were determined using the standard addition method. Regarding each metal analysis, a calibration curve was made with 8 standards values. The ranges quantified for each metal were as follows: 1.0×10^{-5} & 1.0×10^{-2} mg.l^{-1} for **Cd**; 5.0×10^{-3} & 1.0 mg.l^{-1} for **Pb**; and 1.0×10^{-5} & 3.0×10^{-2} mg.l^{-1} for **Ni**. Samples were re-analyzed when the correlation coefficient for the calibration of eight standards was < 0.99. Blank solutions were prepared for each type of sample, following the respective sample treatment for quality assurance purposes.

All the quantifications were done with blank and samples blank (HNO_3 1%) procedure run in parallel. For each sample, the sample blank was subtracted; mean values and respective standard deviations were calculated (Carvalho *et al.*, 2011; Figueira *et al.*, 2011; Pedro *et al.*, 2013).

Different legislation was consulted and for each metal the maximum values authorized on molluscs and water (surface water, water for consumption and wastewater) were noted (**Table 2.1**).

Table 2.1: Maximum values accepted by different legislation for the pollutants Cd, Pb and Ni in molluscs and in different types of water.

Metals			**Cd**	**Pb**	**Ni**
Mollusc Max. levels (mg.kg^{-1}, w.w.)	EFSA / Regulation (EC) No 1881/2005		1.0	1.5	-
Inner surface waters Max. levels (mg.l^{-1})	EFSA / Directive (EC) 2008/105/CE DL n°103/2010		0.00025 0.00025	0.0072 0.0072	0.02 0.02
Max. value on wastewater discharges (mg.l^{-1})	DL n°236/98	ELV	0.2	1.0	2.0
Water quality for Humans consumption (mg.l^{-1})	DL n°236/98	MAV	0.005	0.05	0.05

EFSA - European Food Safe Authorities; **ELV** – Emission Limit Value for residual water discharges; **MAV** – Maximum Admissible Value; **w.w.**- wet weight.

2.5. Data analysis

2.5.1. Physiological Responses

2.5.1.1. Biometric characterization

For each sampling moment, six cockles collected on each sampling station were characterized biometrically. Carefully, each cockle was defrosted and maximum measures to their length (from the anterior to the posterior margin) and height (from the umbones to the ventral margin) were determined, using a calliper rule. To define their weight (w), each cockle was weighted by their wet-weight (w.w.) with/out valves and, after 48h of desiccation in a laboratory oven (Memmert DryingUFB 500, Memmert Germany) at 60°C, by their dry weight (d.w.) with/out valves.

2.5.1.2. Bioaccumulation factor determination (BAF)

For each sampling occasion, the bioaccumulation factor (BAF) of each animal was calculated by the ratio between the total concentration of a given element in the organism tissues by the concentration of that element in the sediment (Figueira *et al.*, 2011; Zhao *et al.*, 2013; Pedro *et al.*, 2013; Velez *et al.*, 2015) using the following equation:

$$\boldsymbol{BAF = \frac{Cx}{Cs}}$$

Where BAF is the BAF calculated using empirical data ($1kg^{-1}$of tissue)*;* Cx is the concentration of the element (Cd, Ni or Pb) in the soft tissue of the cockle ($mg.kg^{-1}$, dry weight); and Cs is the concentration of the same element in the sediment ($mg.kg^{-1}$, dry weight) (Pedro *et al.*, 2013; Zhao *et al.*, 2013).

2.5.1.3. Calculus of the concentration factor (CF)

Once the metals concentrations on cockles and seawater samples were determined, the concentration factor (CF) was calculated using the following equation:

$$\boldsymbol{CF = \frac{Co}{Csw}}$$

Where CF is the concentration factor express on $l.kg^{-1}$, always referred to the soluble fraction in seawater*;* Co is the concentration of the element (Cd, Pb or Ni) in the soft tissue of the cockle ($mg.kg^{-1}$, dry weight); and Csw is the concentration of the same element in the seawater ($mg.l^{-1}$ dissolved) (Conti *et al.*, 2011).

2.5.2. Dietary risk assessment

Since *C. edule* is widely consumed as seafood by humans, a metal dietary risk assessment was performed. The element content in the whole soft parts of the cockles allowed the calculation of the mass of bivalves necessary to be consumed per week by a 70kg adult so as not exceed the Provisional Tolerable Weekly Intake (PTWI) fixed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Figueira *et al.*, 2011; Zhao *et al.*, 2013; Velez *et al.*, 2015).

2.5.3. Statistical analyses

The statistical software (IBM® SPSS® Statistics 22) was used for all the univariate statistical analyses. Prior to any statistical analyses, all the data were first tested for normality (using non-parametric test *Kolmogorov-Smirnov*) and homogeneity of variance to meet statistical demands, and transformed whenever necessary. However, if the transformations did not remove heterogeneity, analyses were performed on the untransformed data, since analysis of variance is quite robust to departures from their assumptions (Underwood, 1997).

Two-Way ANOVA analyses were used to test the effects of the sampling station, of the seasons and, in specific situations, of the years on the physicochemical data. Also, the effects of the sampling station and of the seasons, on the presence of each metal contaminant on environmental and biological samples and on the cockle's physiological responses (Biometry, BAF and CF), were tested following the same techniques. The results were considered significant when $\rho < 0.05$, and the significant effects detected were then subjected to post-hoc tests: (i) Tukey HSD to analyse the individual effects of the factors; (ii) Bonferroni tests to analyse the significant interactions between the factors.

Due to the non-normal behaviour of several variables and the failure of transformations to remove their heterogeneity, spearman's correlation ($\rho_{spearman}$) was used to test if the presence of the contaminants on the bivalves' tissues was correlated with their presence on the water (dissolved) and on the sediment samples, via BAF and CF estimations (significance level ρ-value < 0.05). Other correlations analysed, following the same procedure, were the relationship between the cockle's contaminants data and the cockle's biometrics data (length, height, dry weight). Finally, it was tested the relationship between all the physicochemical parameters.

3. RESULTS

3. Results

3.1. Physicochemical characterization of sampling sites

During the study period, the water at ML station presented higher temperature and salinity values (on average 20.6ºC; 27.4‰) than BSB (on average 18.2ºC; 11.8‰) (**Figure 3.1**). These observations were confirmed by the significant differences detected by the ANOVA analyses between stations for both temperature and salinity (ρ-value < 0.05) (**Table 3.1**).

For both stations, the water temperature was lowest on winter (09 & 10) as compared to the other seasons (**Figure 3.1 A**). In 2009, and as expected, the temperature increased accordingly to the season of the year. However, in 2010, summer appeared to be a little less warm than spring, especially for the ML station. The Two-Way ANOVA analysis proves that the seasons have a significant influence on the variation of water temperature. Namely on winter 09 & 10 that have a lower temperature than some seasons and spring 09 which presents a lower temperature than summer 09 (ρ-values < 0.05) has revealed by the Tukey HSD tests (**Table 3.1**).

As mentioned before, the salinity values were always higher at the ML station and were between 30 - 35‰, except on two seasons: winter 10 (15.8‰) and spring 10 (15.3‰). For the BSB station the values observed were less stable and show a larger range, oscillating between 0.48‰ (winter 10) and 31.3‰ (summer 09). Another possible observation on both stations was that, in general, on year 2009 the salinity seems to be higher as compared to the year 2010 (on average 25.0‰ as opposed to 12.3‰, respectively; **Figure 3.1 B**). In fact, although significant differences on the salinity between seasons were not detected by the ANOVA, this technique confirmed a significant difference between the years, with the year 2009 having higher salinities than 2010 (**Table 3.1**).

The pH values for ML station were 8.3 on the majority of the seasons, except on spring 09 (8.7) and winter 10 (9.2). For the BSB station, the pH values were once again less stable than for the ML station and have been a ranged between 7.7 (spring 10) and 8.7 (spring 09) (**Figure 3.1 B**). The average values were 8.5 and 8.3 at ML and BSB station, respectively, however no significant differences on pH were observed among the stations neither between the seasons or years (ρ-value > 0.05).

Relatively to precipitation, for both stations, the season winter 09 (73.6 mm) was less rainy than winter 10 (186.2 mm) (**Figure 3.1 C**). As expected, summer was the driest season on both years (summer 09: 11.9 mm; summer 10: 0 mm). Precipitation on spring 10 was almost the double than spring 09, respectively 72.90 mm and 35.6 mm. In a global analyses, the year 2009 (average 41.65 mm) seems to have been less rainy than the year 2010 (average 86.4 mm). Nevertheless, no significant differences on precipitation were observed between seasons neither between years (ρ-value > 0.05).

Table 3.1: Two-Way ANOVA and post-hoc test results for physicochemical characterization of sampling sites at the Óbidos Lagoon during the study period. Only the variables that presented significant results were represented (ρ-value < 0.05). It was considered the effects of stations (ML and BSB), seasons (winter, spring, summer, autumn 2009 and winter, spring, summer 2010) and years (2009 and 2010) as factors. df – degrees of freedom; MS – mean square.

ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**ρ-value**
Temperature				
Stations	1	20.09	17.056	0.006
Seasons	6	32.25	27.362	0.000
Salinity				
Stations	1	847.09	16.350	0.007
Years	1	550.131	7.790	0.018

Post-hoc tests			
Source of variation	**Test**	**Condition**	**ρ-value**
Temperature			
Seasons	Tukey HSD	Comparison:	
		Winter 09 and Summer 09	0.001
		Winter 09 and Autumn 09	0.002
		Winter 09 and Spring 10	0.005
		Winter 09 and Summer 10	0.010
		Spring 09 and Summer 09	0.017
		Winter 10 and Spring 09	0.049
		Winter 10 and Summer 09	0.001
		Winter 10 and Autumn 09	0.002
		Winter 10 and Spring 10	0.005
		Winter 10 and Summer 10	0.010

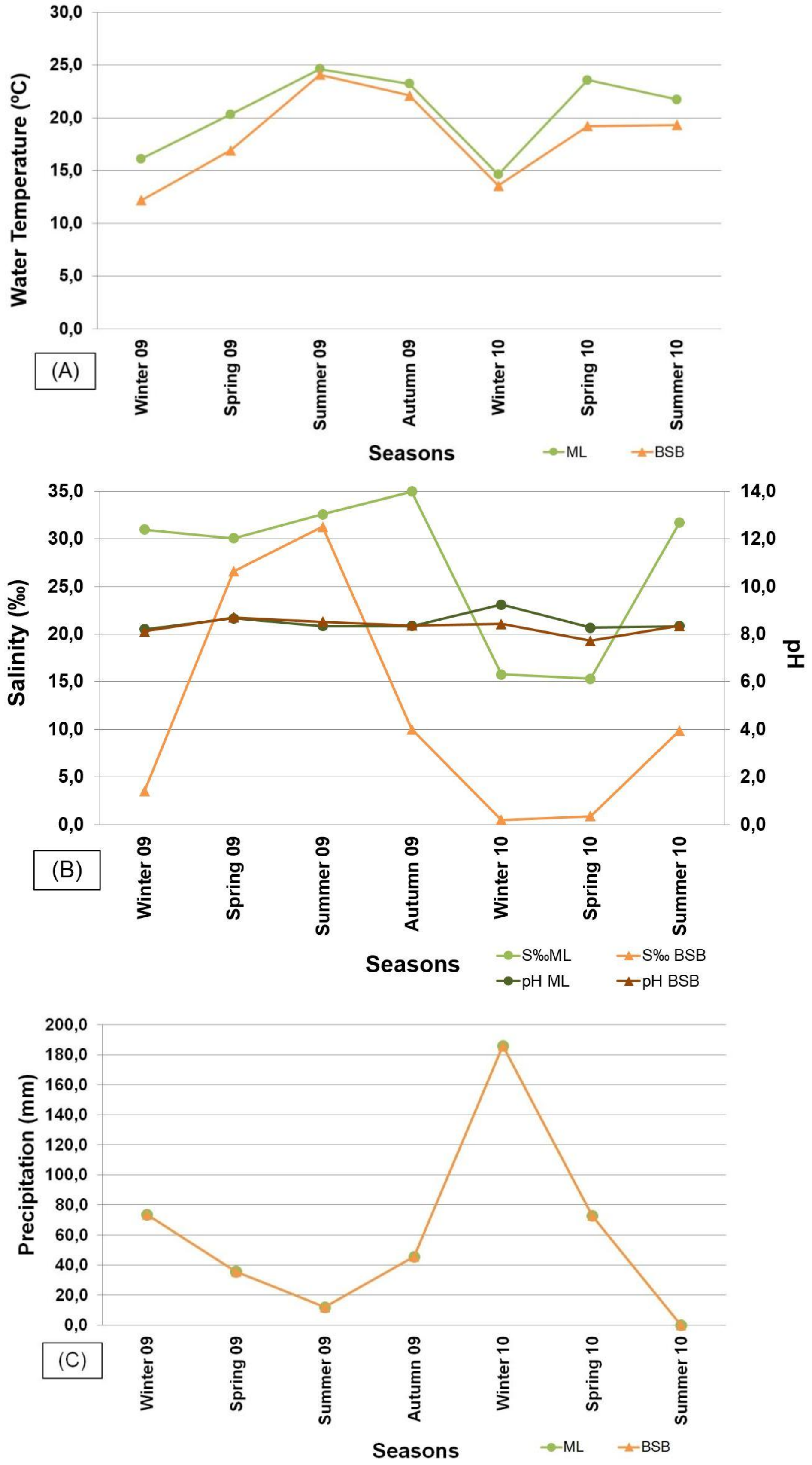


Figure 3.1: Physicochemical parameters at the Middle Lagoon (ML) and at the *Bom Sucesso* Branch (BSB) stations during the study period at the Óbidos Lagoon. (A) Water Temperature (ºC), (B) Salinity (‰) and pH, (C) Precipitation (mm), according to SNIRH data.

The variation of the content on organic matter (OM, %) determined on the surface sediments samples during the study period was, in general, similar between both stations (**Figure 3.2**). However, BSB station registered a higher average value of organic matter content as opposed to the ML station (OM = 0.967%, OM = 0.384%, respectively). On both stations, a seasonal or annual pattern of variation of this parameter was not observed, and significant differences between stations and seasons were not detected by the statistical test (ρ-value > 0.05).

Nevertheless, it is important to highlight the high value of OM that occurred on season winter 09 for the station BSB, with a value of OM = 4.471 ± 2.41% as an opposite to OM = 0.507 ± 0.06% for ML station.

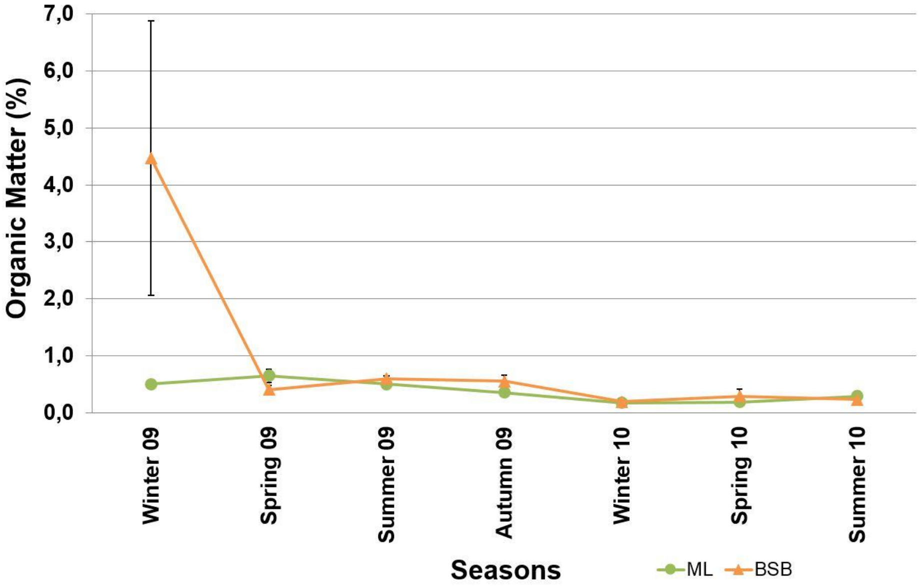


Figure 3.2: Organic matter content (%) of the sediments at the Middle Lagoon (ML) and *Bom Sucesso* Branch (BSB) stations during the study period at the Óbidos Lagoon. Values are the mean of 3 replicates. Error bars correspond to standard deviations.

Finally, it was tested the Spearman's correlation technique ($\rho_{spearman}$) among all the physicochemical parameters. The results suggest 2 significant relationships (ρ-value < 0.05). One, between the variables temperature and salinity, associated with a positive modest correlation ($\rho_{spearman}$ = 0.613 with ρ-value = 0.02). Thus, when temperature increased, salinity increased too. Another modest relationship was found between temperature and precipitation ($\rho_{spearman}$ = - 0.638 with ρ-value = 0.014). Now, a negative modest correlation so when precipitation increased the water temperature decreased and vice versa.

3.2. Presence of metals

3.2.1. Environmental Samples

In the present study the metal concentrations of Cadmium, Lead and Nickel on environmental samples, namely water and sediment samples, on two sites of the Óbidos Lagoon were determined. The water samples allowed to assess the metals concentrations dissolved and suspended on the water, while the sediment samples allowed to estimate the metals concentrations accumulated on the bottom of the Óbidos Lagoon.

3.2.1.1. Cadmium

The Cd element was detected in both fractions of the water samples (dissolved and suspended) analysed, but not on the sediments (**Figure 3.3**).

As can be seen, during the study period Cd was only detected dissolved in the water column in one occasion and at the ML station in summer 09 ($0.002mg.l^{-1}$; **Figure 3.3 A**). No significant differences were observed in terms of dissolved Cd concentration among the stations or the seasons, neither between years (ρ-value > 0.05).

The analysis of the suspended fraction of the waters seems to demonstrate that on ML station Cd appears recurrently on summer seasons, since the value on summer 09 was $0.017mg.l^{-1}$ and on summer 10 it was higher $0.021mg.l^{-1}$(**Figure 3.3 B**). Furthermore, for this station, Cd was also detected on winter 10 and in a much higher concentration (Cd = $0.041mg.l^{-1}$). In fact, the presence of Cd in the suspended fraction in winter 10 was also detected at the BSB station and in similar concentrations (Cd = $0.043mg.l^{-1}$). It is important to refer that at BSB station this was in fact the only identified Cd occurrence along the study period. The ANOVA tests revealed that the seasons affect significantly the suspended Cd concentration, with winter 10 presenting higher Cd concentrations than all the other seasons (ρ-value < 0.05) (**Table 3.2**).

Another pattern can be observed, in fact, the suspended Cd contamination seems to be more frequent on year 2010 than 2009, although no significant differences were found (ρ-value < 0.05).

Table 3.2: Two-Way ANOVA and post-hoc test results for suspended Cd concentration considering the effects of stations (ML and BSB), seasons (winter, spring, summer, autumn 2009 and winter, spring, summer 2010) and years (2009 and 2010) as factors. Only the variables that presented significant results were represented (ρ-value < 0.05). df – degrees of freedom; MS – mean square.

ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**ρ-value**
[Cd]				
Seasons	6	0.000	10.273	0.006
Post-hoc tests				
Source of variation	**Test**	**Condition**		**ρ-value**
[Cd]				
Seasons	Tukey HSD	Comparison:		
		Winter 10 and Winter 09		0.008
		Winter 10 and Spring 09		0.008
		Winter 10 and Summer 09		0.023
		Winter 10 and Autumn 09		0.008
		Winter 10 and Spring 10		0.008
		Winter 10 and Summer 10		0.030

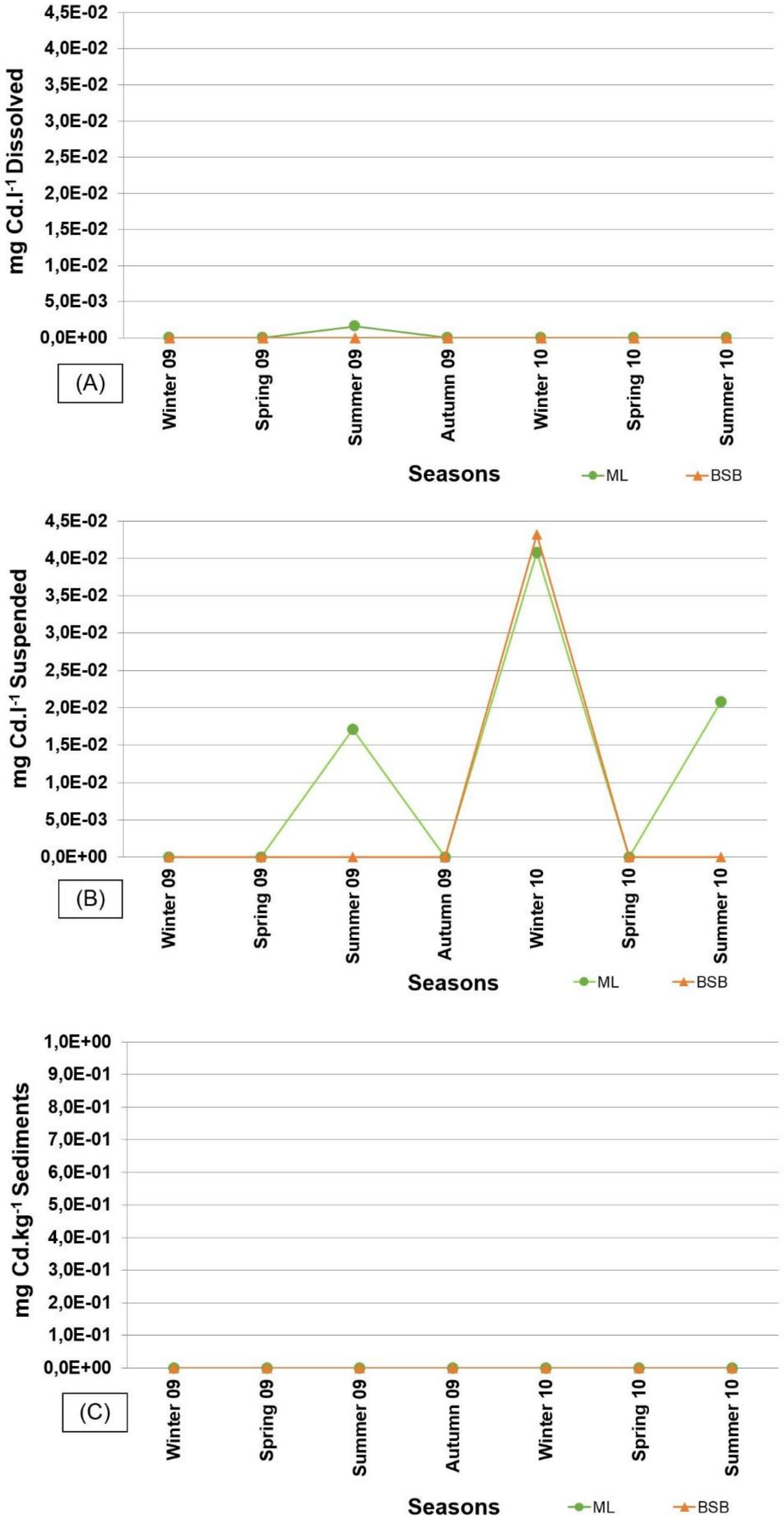


Figure 3.3: Metal concentrations of Cadmium (Cd) at the Middle Lagoon (ML) and *Bom Sucesso* Branch (BSB) stations during the study period at the Óbidos Lagoon. (A) Total Cd dissolved in the water column, in milligrams per liter; (B) Total Cd fraction suspended in the water column, in milligrams per liter and (C) Total Cd in sediments, in milligrams per kilogram, express in dry wet.

3.2.1.2. Lead

The Pb element was detected in both the sediment and water samples analysed, including the dissolved and suspended fractions of the water samples, and in all occasions (**Figure 3.4**).

It was not detectable a particular pattern of variation on dissolved Pb water samples at the stations or seasonally during the study period (**Figure 3.4 A**), and significant differences between seasons and stations were in fact not detected by the ANOVA procedures. However, a global analysis of the presence of dissolved Pb in the water samples shows that in the year 2009 the concentrations of Pb dissolved in water were higher as compared to 2010 (on average 0.120 mg.l^{-1} as opposed to 0.026 mg.l^{-1}, respectively). According to the ANOVA results, the year 2009 was significantly more polluted with dissolved Pb than the year 2010 (df = 1; MS = 0.03; Z = 8.683; ρ value = 0.015).

The concentration of Pb suspended in the water samples revealed a similar behaviour for both stations and along the seasons. The ML station appears to present higher metal concentrations in all the seasons, except for winter 09 (**Figure 3.4 B**) though; statistically no significant differences were demonstrated between stations. Relatively to the effect of seasons on suspended Pb water samples, winter 10 appears more polluted than the others seasons. Effectively, the ANOVA analysis shows that seasons influenced suspended Pb concentrations, namely between winter 10 and summer 10 (df = 5; MS = 0.111; Z = 5.179; ρ value = 0.048; Tukey HSD test: ρ value = 0.047), where the first has an higher contamination than the second. Between year 2009 and 2010 it was not observed a particular pattern, and significant differences were not found.

Regarding the presence of Pb in the sediments, the BSB station seems to be more contaminated than the ML station presenting higher metal concentrations in most occasions (on average 364.5 mg.kg^{-1} as opposed to 257.7 mg.kg^{-1}, respectively; **Figure 3.4 C**). For the BSB station Pb contaminations were above 400 mg.kg^{-1} on the majority of the seasons of the study, except on spring 09, summer 09 and winter 10. Comparatively, ML station was characterized with Pb contaminations above 250 mg.kg^{-1} but below 400 mg.l^{-1} on most periods of the study, except in both winters and in summer 10. Annually, a specific pattern of contamination was not observed in any of the stations. Despite these observations, statistically significant differences on the Pb concentration detected on the sediment samples between stations, seasons or annually were not shown.

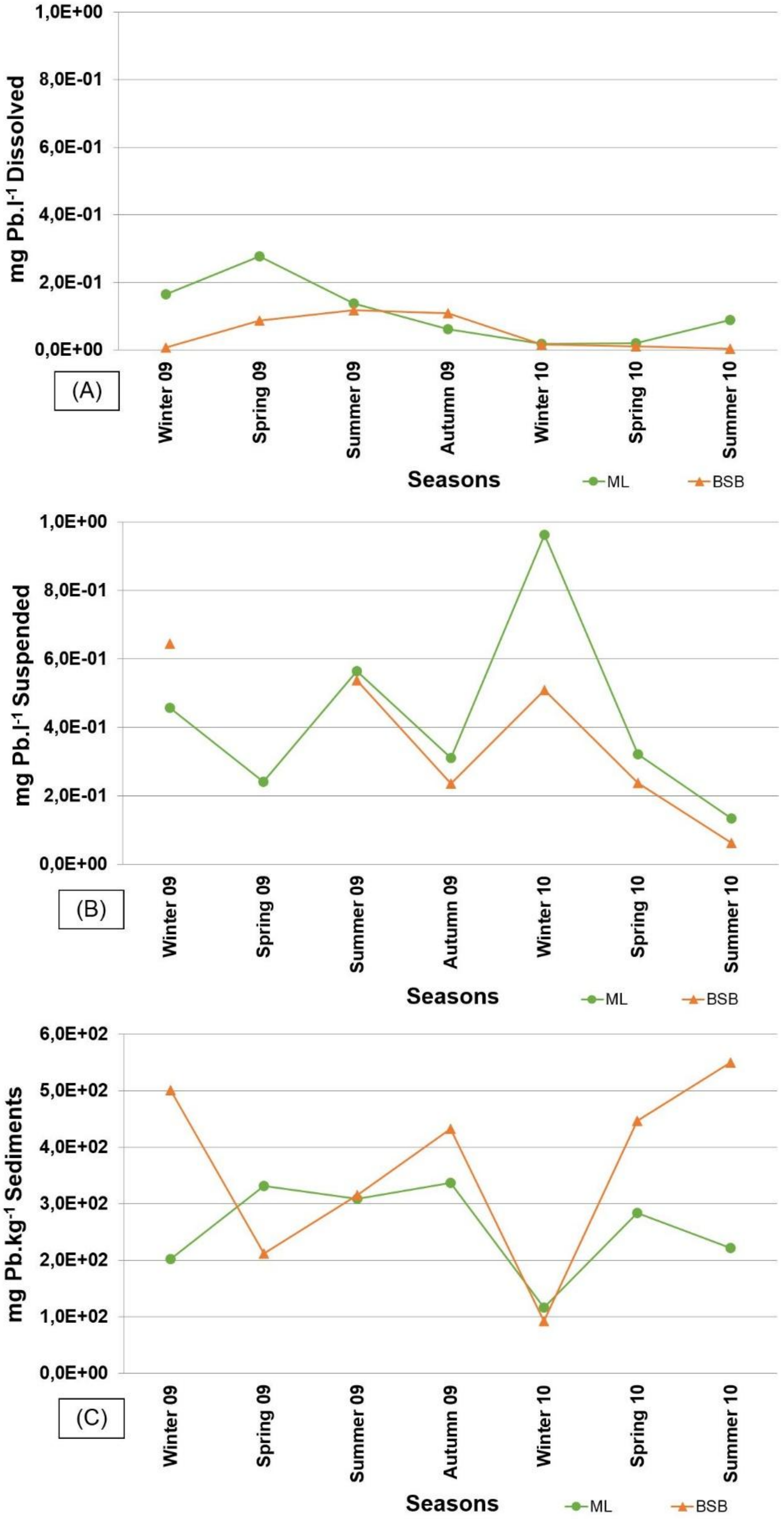


Figure 3.4: Metal concentration of Lead (Pb) in the Middle Lagoon (ML) and at the *Bom Sucesso* Branch (BSB) stations during the study period at the Óbidos Lagoon. (A) Total Pb dissolved in the water column, in milligrams per liter; (B) Total Pb fraction suspended in the water column, in milligrams per liter; (C) Total Pb in the sediments, in milligrams per kilogram, express in dry wet.

3.2.1.3. Nickel

During this study, the metal Ni was only detected on the water samples and predominantly on the dissolved form, at both stations (**Figure 3.5**).

Regarding the dissolved fraction of the water, Ni was detected most often on the ML station, namely on all the seasons of year 2009 and on summer 10 (0.0289 mg.l^{-1}). For the BSB station, Ni was only detected on autumn 09 (0.009 mg.l^{-1}) and on summer 10 (0.0293 mg.l^{-1}). The season summer 10 presented the highest values registered for both stations (**Figure 3.5 A**). The Two-Way ANOVA analysis shows that no significant differences were found in terms of dissolved Ni contamination between the stations and the years (ρ-value > 0.05). However, the same analysis showed a significant difference on the dissolved Ni contamination on seasons namely on summer 10 with all the other seasons (**Table 3.3**).

For the suspended fraction of the water samples, the presence of Ni was identified only on winter 10 at the BSB station ([Ni] = 0.0009mg.l^{-1}) - **Figure 3.5 B**. No significant differences were shown.

Table 3.3: Two-Way ANOVA and post-hoc test results for dissolved Ni concentration considering the effects of stations (ML and BSB), seasons (winter, spring, summer, autumn 2009 and winter, spring, summer 2010) and years (2009 and 2010) as factors. Only the variables that presented significant results were represented (ρ-value < 0.05). df – degrees of freedom; MS – mean square.

ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**ρ-value**
[Ni]				
Seasons	6	0.000	39.862	0.000
Post-hoc tests				
Source of variation	**Test**	**Condition**		**ρ-value**
[Ni]				
Seasons	Tukey HSD	Comparison:		
		Summer 10 and Winter 09		0.000
		Summer 10 and Spring 09		0.000
		Summer 10 and Summer 09		0.000
		Summer 10 and Autumn 09		0.001
		Summer 10 and Winter 10		0.000
		Summer 10 and Spring 10		0.000

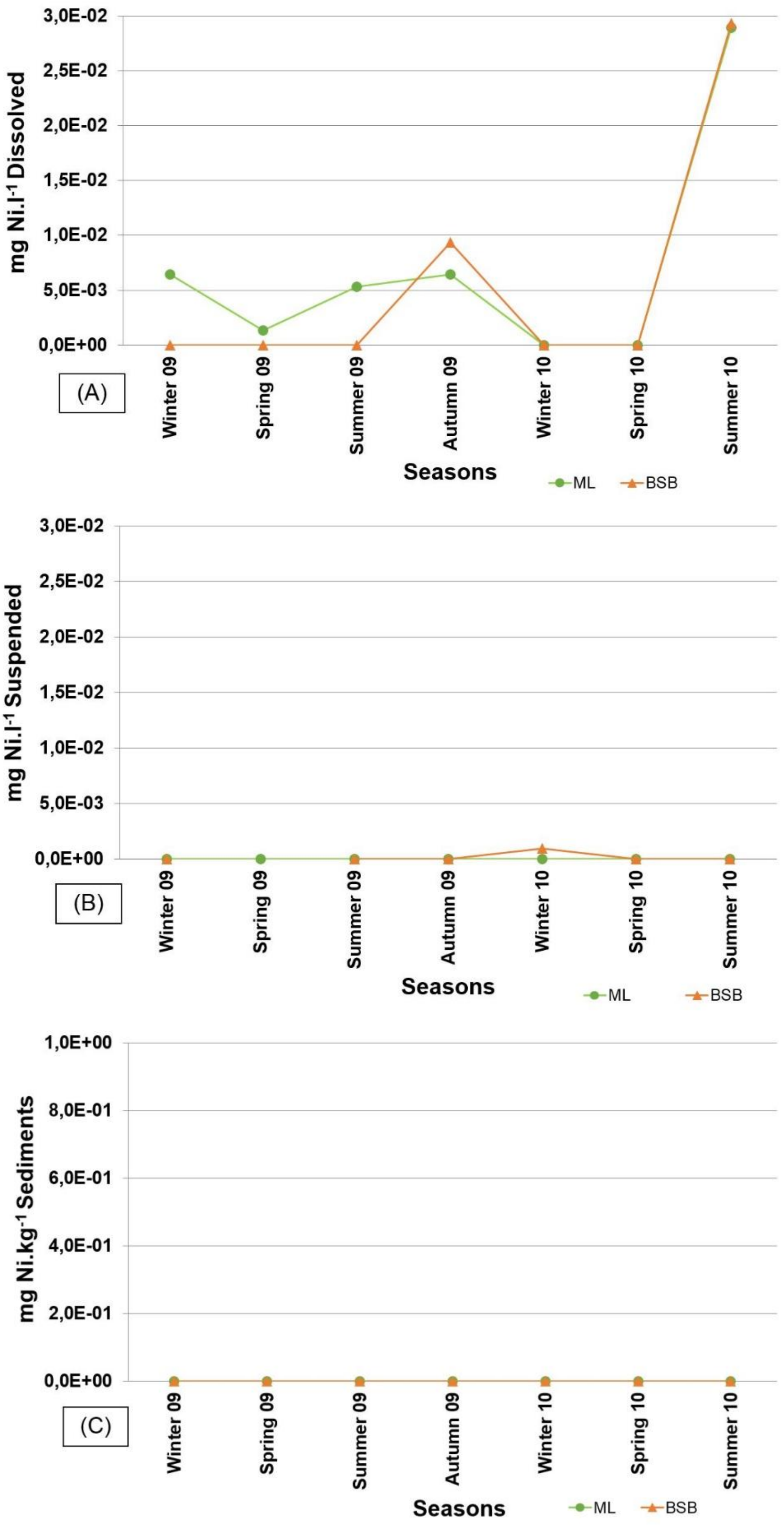


Figure 3.5: Metal concentration of Nickel (Ni) in the Middle Lagoon (ML) and at the *Bom Sucesso* Branch (BSB) stations during the study period, at the Óbidos Lagoon. (A) Total Ni dissolved in the water column, in milligrams per liter; (B) Total Ni fraction suspended in the water column, in milligrams per liter; (C) Total Ni in sediments, in milligrams per kilogram, express in dry wet.

3.2.2. Biological samples – Cockles

The metals assessment on the biological samples of *C. edule* revealed that all the 3 metals (Cd, Pb and Ni) analyzed were detected at least once. Contamination with metal Cd was identified on only one cockle's sample on winter 09 at ML station. Contamination with the metals Pb and Ni on cockle's samples was more recurrent and it was possible to observe more samples contaminated with Ni than Pb (**Table 3.4**).

At the ML station, Pb contaminated cockles were identified in 5 of the 7 sampling moments namely, spring 09, summer 09, winter 10, spring 10 and summer 10 (**Table 3.4**). However, at the BSB station, cockles contaminated with Pb were only observed in 3 moments (spring 09, autumn 09 and summer 10, **Table 3.4**). So, it seems that cockles at ML station were more polluted with Pb than at BSB station. In the global, the results suggest that the cockles were more polluted on the year 2010 than in 2009 (on average 11.230 mg.kg^{-1}, dry weight as opposed to 7.592 mg.kg^{-1}, dry weight, respectively). However, it wasn't identified any significant difference neither between stations nor seasons, or years.

Table 3.4: Seasonal variation of metal concentration (Cd, Pb, Ni; in milligrams per kilogram dry weight) in *C. edule*'s tissues for each station, Middle Lagoon (ML) and *Bom Sucesso* Branch (BSB), during the study period. Values are the mean of 3 replicates, except "*" summer 09 for ML station with N=2.

		Cd		**Pb**		**Ni**	
Seasons	**Stations**	**mg.kg^{-1}, (d.w.)**		**mg.kg^{-1}, (d.w.)**		**mg.kg^{-1}, (d.w.)**	
Winter 09	ML	0.107	±0.19	0	-	1.220	±0.59
	BSB	-	-	-	-	-	-
Spring 09	ML	0	-	40.580	±62.90	3.263	±0.44
	BSB	0	-	4.831	±2.08	6.198	±10.14
Summer 09	ML*	0	-	1.997	±0.04	5.596	±3.28
	BSB	0	-	0	-	-	-
Autumn 09	ML	0	-	0	-	6.434	±8.75
	BSB	0	-	7.681	±13.30	3.207	±.2.43
Winter 10	ML	0	-	44.851	±9.75	7.013	±.2.63
	BSB	0	-	0	-	11.368	±0.81
Spring 10	ML	0	-	0.129	±0.22	0.723	±0.36
	BSB	0	-	0	-	3.263	±4.55
Summer 10	ML	0	-	8.314	±7.18	4.441	±0.95
	BSB	0	-	13.136	±8.16	15.424	±4.37

The Two-Way ANOVA results of the contamination with Ni on cockle´s samples at BSB station (average 8.819 mg.kg^{-1}, dry weight) were significantly different from the ML station (average 3.986 mg.kg^{-1}, dry weight), proving that at BSB station the animals were more contaminated with Ni than at ML station (df = 1; MS = 167.25; Z = 5.613; ρ-value = 0.026). Nevertheless, it was not identified a seasonal or annual effect on the contamination with Ni.

3.3. Physiological responses

For each station, physiological responses of *C. edule* were studied to determine if the metal contamination presented specific consequences on some aspects of the development of this bivalve in their natural environment.

3.3.1. Biometric characterization

Regarding the length of the *C. edule* specimens from the ML station it seems to have a smaller length than the organisms from the BSB station (on average 21.77 mm as opposed to 23.34 mm, respectively). Relatively to a seasonal effect, it was observed that the organisms have a higher length on spring 09 and summer 10. It was shown also that, on all the seasons of 2009, the length of the specimens on BSB station was higher than ML and, on all the seasons of 2010 it was the opposite (**Figure 3.6 A**). According to the ANOVA results the exposure of cockles to different stations and seasons had a significant effect on the length of the specimens (ρ-value = 0.000, **Table 3.5**). So, on ML station, cockles on summer 10 have a significantly higher length than the cockles on autumn 09 and winter 09. Concerning the BSB station, cockles with significant higher length were found on spring 09 as compared with the other seasons (Bonferroni tests results, **Table 3.5**).

Concerning the shells' height, the bivalves from the ML station seemed to present higher heights (average H = 22.2mm) than the animals from the BSB station (average H = 21.4 mm). However, on BSB station there was a height value that stand out from all the other moments, namely spring 09 where cockles have a higher height than the others seasons and station (**Figure 3.6 B**). Once again, the ANOVA results show that the exposure of cockles to different stations and seasons had a significant effect on the height of the organisms (**Table 3.5**). On the BSB station, the cockles had a significant higher height in spring 09 as opposed to the other seasons. This result was also observed in the autumn 09 as compared with the summer 09 and winter 10, at the same sampling station (Bonferroni tests results, **Table 3.5**).

As for the dry weight, the specimens at the ML station presented lower dry weights, on average 0.075 mg, as opposed to 0.180 mg at the BSB station. Once more, on BSB station, the cockles presented higher dry weights in spring 09 comparatively with the other seasons and station (**Figure 3.6 C**). The ANOVA test shows again that the conditions experienced by the bivalves on the different stations and seasons had a significant effect on their dry weights. On ML station, the summer 10 had cockles with a significantly higher dry weight than the organisms on autumn. On BSB station, at season spring 09 it can be found the bivalves with a significant higher dry weight than the other moments. In turn, autumn 09 had cockles with higher dry weight than the summers (Bonferroni test results, **Table 3.5**).

Table 3.5: Two-Way ANOVA and post-hoc test results for biometry characterization of the sampled cockle’s at Óbidos Lagoon during the study period. Only the variables that presented significant results were represented (ρ-value < 0.05). It was considered the effects of stations (ML and BSB) and seasons (winter, spring, summer, autumn 2009 and winter, spring, summer 2010) as factors. df – degrees of freedom; MS – mean square.

ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**ρ-value**
Length				
Stations * Seasons	5	53.33	11.979	0.000
Height				
Stations * Seasons	5	134.57	11.370	0.000
Dry weight				
Stations * Seasons	5	0.027	23.97	0.000
Post-hoc tests				
Source of variation	**Test**	**Condition**		**ρ-value**
Length				
Interaction:				
Stations * Seasons	Bonferroni	<u>ML station:</u>		
		Comparison: Summer 10 and Autumn 09		0.017
		Comparison: Summer 10 and Winter 09		0.025
		<u>BSB station</u>		
		Comparison: Spring 09 and Summer 09, Autumn 09, Winter 10, Spring 10, Summer 10		0.000
Height				
Interaction:				
Stations * Seasons	Bonferroni	<u>BSB station:</u>		
		Comparison: Spring 09 and Summer 09, Autumn 09, Winter 10, Spring 10, Summer 10		0.000
		Comparison: Autumn 09 and Summer 09		0.027
		Comparison: Autumn 09 and Winter 10		0.001
Dry weight				
Interaction:				
Stations * Seasons	Bonferroni	<u>ML station:</u>		
		Comparison: Summer 10 and Autumn 09		0.013
		<u>BSB station:</u>		
		Comparison: Autumn 09 and Summer 09		0.017
		Comparison: Autumn 09 and Summer 10		0.030
		Comparison: Spring 09 and Summer 09, Autumn 09, Winter 10, Spring 10, Summer 10		0.000

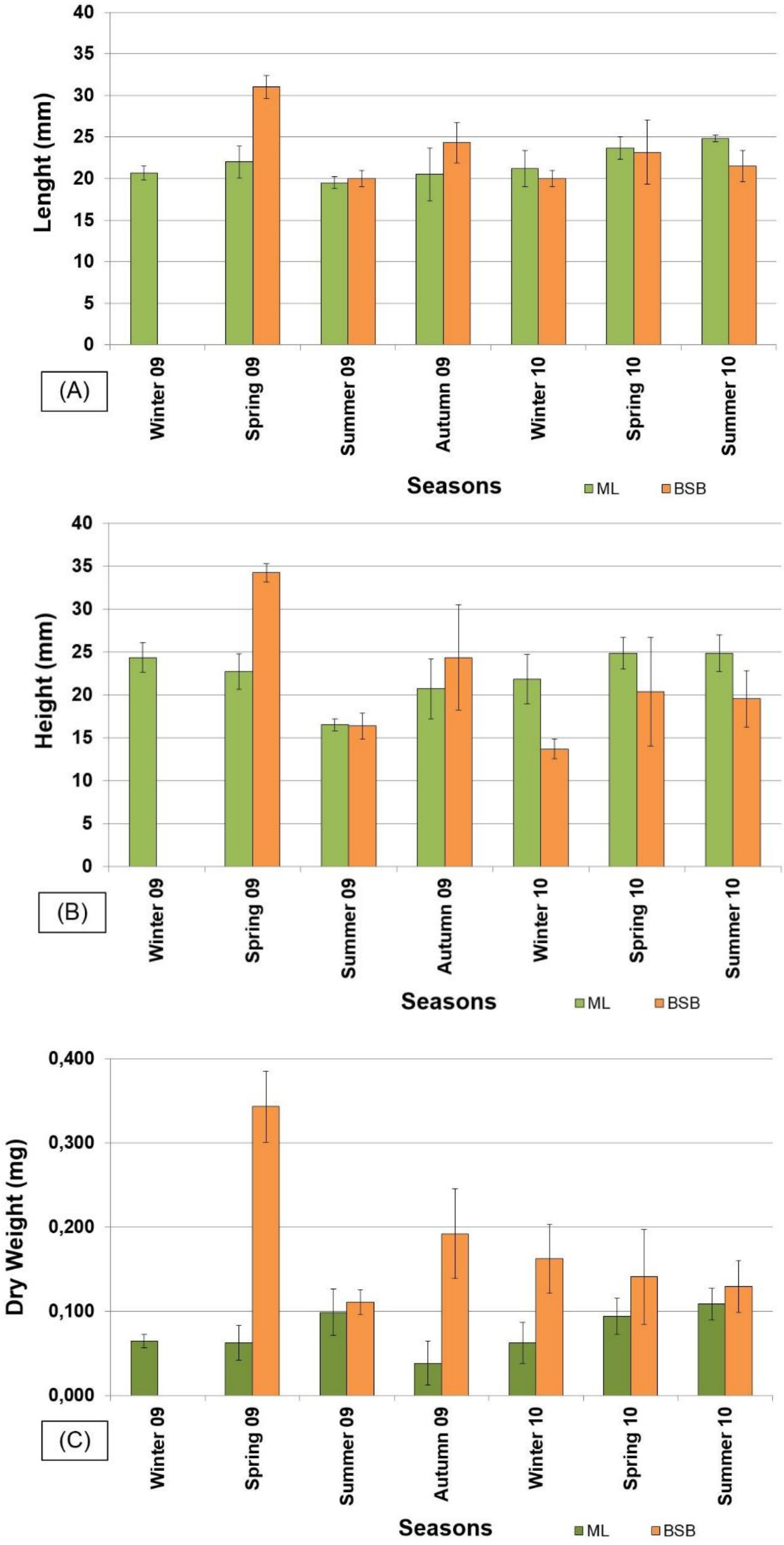


Figure 3.6: Biometric characterization of *C. edule* in Middle Lagoon (ML) and *Bom Sucesso* Branch (BSB) stations during the study period. (A) *C. edule* length; (B) *C. edule* height; (C) *C. edule* dry weight. Values are the mean of N= 6 replicates.

3.3.2. Bioaccumulation factor _ BAF

Since Cd and Ni were not detected on the sediment samples, BAF calculations could only be performed for the contaminant Pb and only in certain seasons.

It was shown that the BAF value from *C. edule* at the ML station (average BAF = 0.071) was higher than from the specimens at the BSB station (average BAF = 0.010). The results did not show a seasonal pattern (**Figure 3.7**). According to the Two-Way ANOVA results, winter 10 on ML station was significantly higher from all the other seasons (df = 5; MS = 0.030; Z = 6.532; ρ-value = 0.000; Bonferroni test: ML station comparison: winter 10 and all the other seasons, except spring 09, ρ-value = 0.000; comparison: winter 10 and spring 09 ρ-value = 0.007).This result demonstrates that both the station and the seasons influenced the Pb bioaccumulation on cockles.

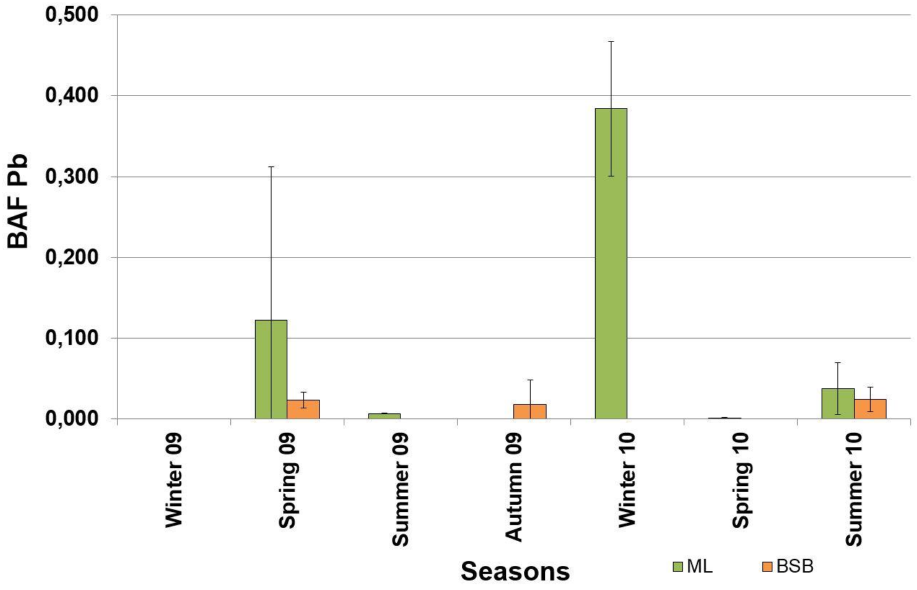


Figure 3.7: Bioaccumulation factor (BAF) of *C. edule*, collected in the Middle Lagoon (ML) and at the *Bom Sucesso* Branch, for the element lead (Pb). Bioaccumulation factor: ratio between total element concentration in the organism, total element concentration in sediments. Values are the mean of 3 replicates, except summer 09 for ML station, N=2. Error bars correspond to standard deviations.

3.3.3. Concentration factor_CF

The Concentration Factor (CF; l.kg^{-1}) was calculated only for Pb and Ni (whenever possible), since Cd occurred only sporadically both on the soluble fraction of the water samples and on the tissues of *C. edule*.

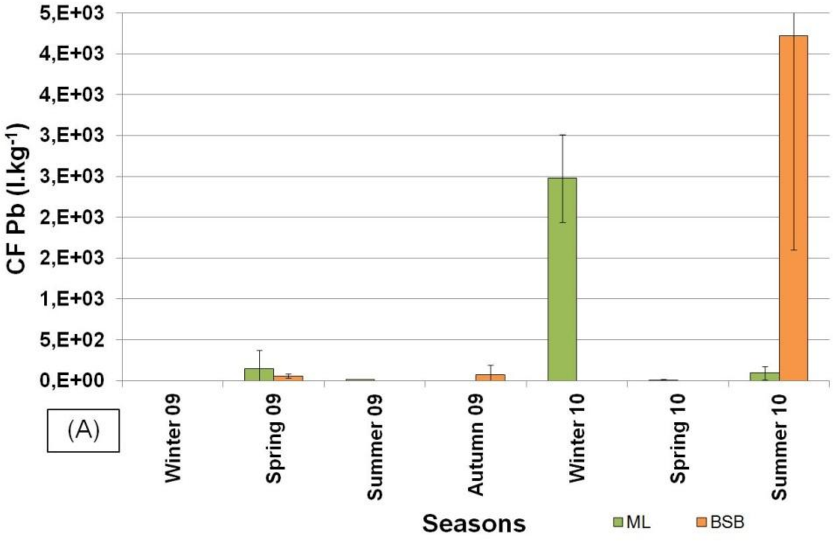
Concerning CF Pb, two high values can be observed. One for winter 10 at the ML station and another for summer 10 at the BSB station (**Figure 3.8 A**).

Regarding CF Ni results, the highest value was obtained on spring 09 at the ML station. Also, another 3 relevant results could be highlighted at the ML station, namely summer 09 > summer 10 > winter 09. At the BSB station, CF Ni were obtained only in 2 occasions, specifically, autumn 09 and summer 10 (**Figure 3.8 B**).

Another observation, it was the fact that at the ML station the CF of both metals could be calculated more often than at the BSB station.

In fact, the influence of the stations and of the seasons on the concentration factor of *C. edule* was demonstrated by the significant differences obtained on the ANOVA analysis. According to this analysis, CF Pb on winter 10 at the ML station and on summer 10 at the BSB station were significantly higher than on the other seasons (Bonferroni tests results, **Table 3.6**).

With reference to CF Ni, the results of this statistical analysis revealed that seasons had a significant effect on CF Ni of *C. edule*, so it was concluded that this factor was higher on spring 09 than on summer 10 and winter 09 (**Table 3.6**).



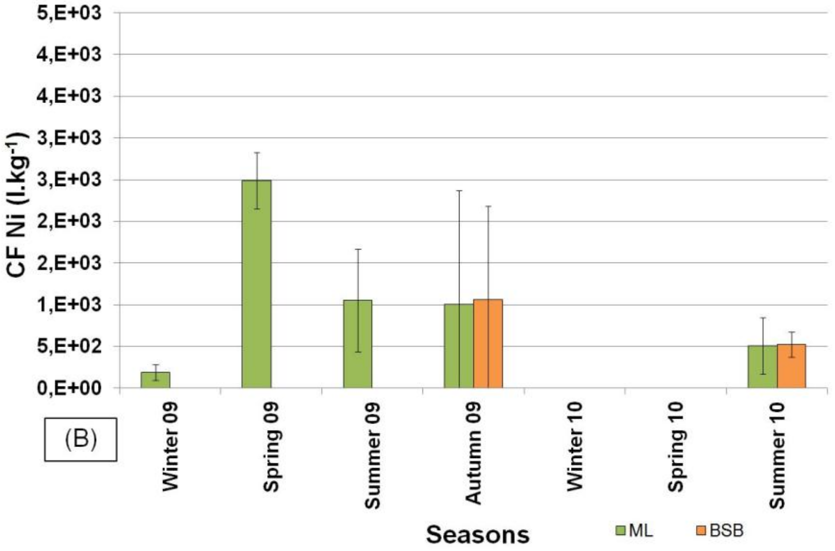


Figure 3.8: Concentration factor (CF) for *C. edule* collected in the Middle Lagoon (ML) and at the *Bom Sucesso* Branch from the Óbidos Lagoon, for the elements: (A) Lead (Pb) and (B) Nickel (Ni). Concentration factor: ratio between total element concentration in the organism / total element concentration in seawater. Values are the mean of 3 replicates, except summer 09 for ML station, N=2. Error bars correspond to standard deviations.

Table 3.6: Two-Way ANOVA and post-hoc test results for CF Pb e CF Ni of sampling cockle's at Óbidos Lagoon during the study period. Only the variables that presented significant results were represented (ρ-value < 0.05). It was considered the effects of stations (ML and BSB) and seasons (winter, spring, summer, autumn 2009 and winter, spring, summer 2010) as factors. df – degrees of freedom; MS – mean square.

ANOVA

Source of variation	df	MS	F-statistic	ρ-value
CF Pb				
Stations * Seasons	5	4.05E+06	11.208	0.000
CF Ni				
Seasons	4	2.369E+06	4.292	0.018

Post-hoc tests

Source of variation	Test	Condition	ρ-value
CF Pb			
Interaction:			
Stations * Seasons	Bonferroni	<u>ML station:</u>	
		Comparison: Winter 10 and Winter 09, Autumn 09,	0.022
		Comparison: Winter 10 and Spring 10	0.023
		Comparison: Winter 10 and Summer 10	0.033
		Comparison: Winter 10 and Spring 09	0.042
		<u>BSB station:</u>	
		Comparison: Summer 10 and Spring 09, Summer 09, Autumn 09, Winter 10, Spring 10	0.000
CF Ni			
Seasons	Tukey HSD	Comparison:	
		Spring 09 and Winter 09	0.014
		Spring 09 and Summer 10	0.015

3.4. Dietary risk assessment

Comparing the metal concentration in cockles with the maximum levels regulated by European Food Safe Authorities (EFSA), United States Food and Drug Administration (USFDA) and Food Standards Australia and New Zealand (FSANZ), it was possible to conclude that contamination with Cd and Pb was above the maximum values, except the minimum value on ML station for Pb contamination. Relatively to Ni contamination, only the maximum contamination detected at the BSB station, was higher than the maximum value regulated by USFDA agencies, almost the double (**Table 3.7**).

From among the three metals in study, the ingestion of cockles per week is very limited for all the metals. Regarding the metal Cd, it seems that on the ML station of the Óbidos Lagoon, the contamination by this element is smaller than by Pb and Ni and the consumption to exceed the PTWI value for an adult of 70kg was 4.587 kg.week^{-1} d.w.. About Pb contamination on both stations, the PTWI was exceed very easily since the contamination with Pb was high. An exception was found on the minimum value on ML station, where it was necessary to consume 13.521kg.week^{-1} d.w. to reach the PTWI. At last, for the metal Ni, once more, the PTWI was exceeded with a consumption of cockles between 0.160 (for the maximum contamination) and3.390kg.week^{-1} d.w. (for the minimum contamination). Curiously, the BSB station represented the worst case, except on Pb where maximum value was observed at the ML station.

Table 3.7: Concentrations of different elements (Cd, Pb, Ni) on edible part of *C. edule* from the Óbidos Lagoon and the amount of cockles that a 70 kg adult needs to consume to exceed Provisional Tolerable Weekly Intake, PTWI.

Metals			**Cd**	**Pb**	**Ni**
Max. levels (mg.kg^{-1} w.w.)	EFSA		1.0	1.5	80.0
	USFDA		4.0	1.7	
	FSANZ		2.0	2.0	
Provisional Tolerable Weekly Intake, PTWI (mg.kg^{-1}.week^{-1})	JECFA		0.007	0.025	0.035
	FSANZ		0.007	0.025	
PTWI (mg70 kg^{-1}.week^{-1}, based on JECFA values)			0.49	1.75	2.45
Metal concentration in cockles (mgkg^{-1}, d.w.)					
Station ML		Max	0.107	44.851	7.013
		Min	0	0.129	0.723
Station BSB		Max	0	13.136	15.424
		Min	0	4.831	3.207
Amount of cockles consumed per week to exceed PTWI (kg. week^{-1}, d.w.)					
Station ML		Max	4.587	0.039	0.349
		Min	-	13.521	3.390
Station BSB		Max	-	0.133	0.159
		Min	-	0.362	0.764

Current consumption guidelines for elements set by different organizations are also presented: **EFSA** - European Food Safe Authorities; **USFDA** - United States Food and Drug Administration; **FSANZ** - Food Standards Australia and New Zealand; **JECFA** - Joint FAO/WHO Expert Committee on Food Additives. **Max. levels** - Maximum levels in mollusc. **w.w.** - Wet weight. **d.w.:** Dry weight.

3.1. The influence of the presence of contaminants in the biological parameters

The effect of the presence of the different contaminants on *C. edule*'s tissues was assessed through the Spearman's correlation technique ($\rho_{spearman}$) and 10 significant (p value < 0.05; $\rho_{spearman} > 0.5$) relationships were found (**Table 3.8**).

As expected, BAF Pb and CF Pb have a positive and very strong correlation with Pb contaminations ([Pb]) on the soft tissues of the cockles (**Table 3.8**). In turn CF Pb and

BAF Pb were positively and very strongly correlated. However, CF Ni has a positive and only modest correlation with Ni contaminations ([Ni]) on the soft parts of the cockles. Surprisingly, a significant positive correlation was found between [Pb] and CF Ni, although it was only modest.

Regarding the biometric variables, the cockles' dry weight was negatively and modestly correlated with [Ni] and CF Ni. As for the height, positive and modest relationships were found with [Pb], BAF Pb and CF Pb. Finally, the length of the cockles exhibited a positive but only modest relationship with their height and dry weight.

Table 3.8: Spearman correlation matrix ($\rho_{spearman}$) for different variables of *C. edule*, namely, length, height, dry weight, metal [Cd], [Ni], [Pb], BAF Pb, CF Pb, CF Ni. Significant correlations at ρ-value < 0.05 are highlighted in bold. (N = 40, except for the correlations between [Ni] and Dry Weight where N =37, and for the correlations involving CF Ni where N = 21)

	Length	**Height**	**Dry Weight**	**[Cd]**	**[Pb]**	**[Ni]**	**BAF Pb**	**CF Pb**	**CF Ni**
Length	1.0								
Height	**0.506**	1.0							
Dry Weight	**0.537**	-0.062	1.0						
[Cd]	0.148	0.056	0.035	1.0					
[Pb]	0.029	**0.628**	**-0.431**	-0.148	1.0				
[Ni]	**-0.441**	0.030	**-0.682**	-0.094	**0.331**	1.0			
BAF Pb	0.079	**0.672**	**-0.398**	-0.148	**0.990**	0.294	1.0		
CF Pb	0.032	**0.614**	**-0.405**	-0.148	**0.972**	**0.370**	**0.962**	1.0	
CF Ni	-0.118	0.131	**-0.654**	-0.111	**0.500**	**0.661**	**0.479**	0.426	1.0

The Spearman correlation technique was also applied between the degree of contamination observed on the environmental samples (dissolved and suspended fractions of the water samples and sediment samples) and on the biological samples, but significant correlations were not detected. However, a positive modest correlation between the $[Ni]_{dissolved}$ on the water samples and the $[Pb]_{suspended}$ on the water samples was found to be significant ($\rho_{spearman}$ = 0.686 with ρ-value < 0.05).

4. DISCUSSION

4. Discussion

The physicochemical parameters, namely, temperature, salinity, pH and organic matter content of the sediments were analysed in the present study to characterize the environmental conditions at the sampling stations. Comparing the water temperature, salinity and pH at the ML station (averages: T = 20.6ºC, S = 27.4‰ & pH = 8.5) with the typical values of standard seawater (S = 35‰ & pH = 8), it is evident a higher influence of tidal cycles than at the BSB station (averages: T = 18.2ºC, S = 11.8‰ & pH = 8.3). The location of the sampling sites in the lagoon, with the ML station located at the middle of the system (right margin), while BSB station is confined at the inner branch of the lagoon (left margin) is also important. All of these observations allow concluding that the ML station suffers a higher influence of the semi-diurnal and fortnight tidal cycles while the BSB station may receive freshwater inputs. This conclusion is in accordance with similar results obtained at the *Barrosa* branch in previous studies by Pereira *et al.* (2009a) or Carvalho *et al.* (2011). The ANOVA analysis supports the previous conclusion, showing that the water temperature and the salinity were significantly different between both sampling stations.

The semi-enclosed characteristic of this lagoon makes this ecosystem more sensitive to freshwater inputs. Comparatively to other coastal zones, spatial and temporal fluctuations in physical (temperature), chemical (salinity, nutrients) and biological (primary and secondary food chains) properties in this type of ecosystems are wider (Crivelli *et al*., 1995). Precipitation increases the entry of freshwater on the lagoon, inducing a decrease of salinity and of the water temperature. The terrestrial runoffs increase the entry of nutrient inputs namely, organic matter, nitrogen and phosphorus to the lagoon and, consequently, an increase of pH occurs (Crivelli *et al*., 1995). Furthermore, the results of the present study show that temperature was directly linked with salinity ($\rho_{spearman} = 0.613$ with ρ-value < 0.05) and with precipitation ($\rho_{spearman} = -0.690$ with ρ-value < 0.01). So, when precipitation has increased, salinity decreased and water temperature has decreased too. This behaviour was also reflected on the significant seasonal variations detected for the temperature and salinity parameters (statistical test).

Since, the BSB station it is farther located from the narrow inlet than the ML station, the input of freshwater by runoff, by precipitation or yet by the *Ditch of Ameal* ($0.08m^3s^{-1}$), the *Arnóia* / *Real* river ($3m^3.s^{-1}$) affects this site even more dramatically than the ML station (ICN *et al.*, 2005; Pereira *et al.,* 2009a,b; Pereira *et al.*, 2010).

The results obtained for salinity and pH in winter 10 and spring 10, on both sampling stations, can be explained by the high precipitation levels that occurred on these seasons. And still, the high values of salinity (31.3‰) observed at BSB on summer 09 can be explained by the low precipitation and by the reduction on the freshwater inputs. In fact, according to some authors (Crivelli *et al*., 1995; Pereira *et al.*, 2009c), the increases in salinity in summer may occur because the evaporation in this site wasn´t compensated by the freshwater inputs (by precipitation or effluents).

The infaunal communities are in direct contact with the sediment, where organic matter and other pollutants are accumulated (Carvalho *et al.*, 2006), so the organic matter content of the sediments was assessed on each station for each season. Although significant differences between the stations or seasons were not detected by the Two-Way ANOVA test, the ML station appeared to present lower organic matter contents on the sediments (0,384% ± 0.4 on average) than the BSB station (0.967% ± 0.4 on average). This fact can be due to the nutrient residence time and the composition of the sediment on each station. These hypotheses were supported by some studies that demonstrated higher nutrient loads and weak tidal currents in inner branches that provide a longest residence time of water, namely 24-26 days for the BSB station, in comparison to the middle / lower lagoon (only 1- 4 days), facilitating the accumulation of organic matter in the sediments (Pereira *et al.*, 2009b; Pereira *et al.*, 2009c). Another support may be that in the inner branches the sediments are mostly muddy while at the ML station the sediments are mainly composed by sand banks (Carvalho *et al.*, 2011). According to Carvalho *et al.* (2005), muddy areas are consistently richer in organic matter than sandy areas, as clay particles tend to bind higher quantities of organic matter.

Previous studies have shown that the Óbidos Lagoon is exposed to nonpoint sources of contamination associated with agriculture and livestock (Kowalski, 2009). Freshwater influx arrives at the lagoon by both branches: at the *Barrosa* branch by the *Cal River* and at the *Bom Sucesso* branch by *Vala do Ameal*. Between the lagoon branches flows the *Arnóia / Real* River that contributes about 90% of freshwater influx into the lagoon. (Malhadas *et al.*, 2009a).These freshwater tributaries are also responsible by draining the agriculture fields, and the domestic and industrial effluents to the lagoon (Pereira *et al.*, 2009a, b). As a consequence, eutrophication was already observed in the lagoon (Pereira *et al.*, 2009b) and maximum metal concentrations of Mn, Ni, Cu and Cd were already detected on the sediments of The *Barrosa* branch in summer 2006 (Pereira *et al.*, 2009c).

Regarding, the *Bom-Sucesso* branch, on previous studies, it has been identified as an intermediate hazard area based on the biological responses of a target species - the shore crab *Carcinus maenas* - while the middle lagoon was considered as a reference area (Pereira *et al.*, 2012).

The physiological responses of indigenous organisms, such as *Cerastoderma edule*, at the study site, can be considered good tools for the evaluation of environmental contamination. These indicator species exhibit clear and reproducible responses to contamination (Nilin *et al.*, 2012). For example, just like *C. edule*, mussels are important filter-feeders that, according to the literature, filter 3 to 9 $l.h^{-1}.g$ dry $weight^{-1}$ of the surrounding water, and are considered good metals accumulators (Conti *et al.*, 2011).

On the present study, the global appreciation of the environmental and biological results for the metal contamination has revealed a different behaviour according to the metal tested (Cd, Pb and Ni). All the three metals were detected during the study period at the Óbidos Lagoon. Cadmium was detected only once on the dissolved fraction of the water (summer 09 at ML station), as well as on cockles samples (winter 09 at ML station), and sporadically on the suspended fraction of the water on summers and winter 10, at the ML station, and only on winter 10 at the BSB station. The metal Pb was detected on all the environmental samples and on some biological samples, occurring most frequently at the ML station. Finally for Ni contamination, the water and cockles samples also show contamination with this metal. On the suspended fraction of the water it was detected only once on winter 10 at the BSB station. On the dissolved fraction, the metal was present on both stations, but it was more common at the ML station. On cockle's samples, Ni was detected on all the available samples. So, it can be stated that, both Pb and Ni, were the prevailing metals on the ecosystem during the study period.

In the aquatic environment Cd can exist as a free ion (Cd^{2+}) or as ionic complexes with other inorganic or organic substances. In seawater, the most common forms are chlorine ion complexes, and in freshwater the free hydrated or carbonated ions are the most frequent forms (EFSA, 2011).

Although dissolved cadmium was detected only in summer 09, and at the ML station, the concentration observed (0.002 $mg.l^{-1}$) was higher than the maximum value legislated on the Directive 2008/105/CE (**Table 2.1**). Regarding suspended cadmium, the contamination of water samples was more common, with Cd at the ML station being consistently detected on summers (0.017 $mg.l^{-1}$ in 2009 and 0.021 $mg.l^{-1}$ in 2010) and on winter 10 (0.041 mg.l^{-1}). Considering the same regulated value, it is possible to state that the concentrations of Cd$_{suspended}$ in the water samples detected on these seasons were all much higher than the value legislated. The values detected on summers on water samples can be related with an increase in the use of motorized vehicles (*e.g.* high shellfish fishing activities, motorized nautical sports) at this station, since this element is present in refined petroleum products and on coatings used to paint the boats, causing the suspension and dissolution of Cd on the water.

At the BSB station, the suspended fraction of the water samples revealed to be contaminated only on the winter of 2010 (0.043 mg.l^{-1}), in a similar degree to the ML station and, once again, higher than the legislated value. The high levels of Cd contamination detected on winter 10 on both stations were confirmed with the ANOVA test, since these concentrations were significantly different from the concentrations detected on the other seasons. This event can be explained by the high precipitation that occurred on this season. The increase in freshwater entries may transport large quantities of Cd from the rivers to the lagoon (OSPAR, 2004). Moreover, in winter 10, restructuring works in order to change "A Aberta" on the *Bom Sucesso"* site to the "*Foz do Arelho*" site were occurring at the lagoon, as well as slight dredging works (Ferreira S., personal information). These two events may also have accentuated the Cd contamination on this season at both stations.

The analysis of the *C. edule* samples, revealed the presence of this metal only in winter 09 (0.107mg.kg^{-1}, d.w.), at the ML station. In the present study, metal contamination on *C. edule* was calculated considering dry weight values, while the regulated values are expressed in wet weight. Therefore, a ratio between these two parameters considering the studied samples was determined, and defined as wet weight = 10 times dry weight of the soft tissues (the same reasoning was used for Pb and Ni comparison). Comparatively with the value fixed by the European Commission Regulation (EC) No 1881/2006 for maximum levels of Cd on bivalve molluscs (1.0 mg.kg^{-1}, w.w.), the contamination detected in *C. edule* samples was higher. May be this value occurred due to the fact that temperature on this season was up than 16ºC, and at this temperature the mollusc increased the flow of water and so increased the uptake of cadmium on their tissues too (OSPAR, 2004). According to the literature, bivalves can accumulate high amounts of pollutants due to their filtration nature, sometimes on higher concentrations than those of the sediments where they are buried (Velez *et al.*, 2015). Moreover, Figueira *et al.* (2011) referred that cockle's bioaccumulated preferentially some metals as Cd than the others at sediments less contaminated.

In the aquatic environment, lead is controlled by many factors, such as pH, salinity, sorption and biotransformation processes. Other parameters, such as sediment lead content, temperature, type and amount of organic matter, also have a significant impact on the lead status in waters. This element occurs in ionic form (highly mobile and bio-available), in organic complexes with dissolved humic materials attached to colloidal particles such as iron oxide or attached to solid particles of clay or dead remains of organisms (EFSA, 2010).

The element Pb was detected on the environmental and on the biological samples. At both stations, all the water samples were contaminated with Pb in the dissolved form. This contamination seemed to be higher on the year 2009 than on the year 2010, fact that was confirmed by a Two-Way ANOVA analysis, as mentioned in the Results section. Therefore, it can be stated that, regarding Pb contamination in the dissolved fraction, the year 2009 (average = 0.120 mg.l^{-1}) was more polluted than 2010 (average = 0.026 mg.l^{-1}). Also, in general, and at both stations, the values detected were higher than the maximum value legislated on the Directive 2008/105/CE (**Table 2.1**).

Just like in the dissolved fraction of the water samples, the presence of Pb in the suspended fraction was confirmed in all the samples. The ML station seems to have a higher degree of Pb contamination than the BSB station, on all the seasons, but no significant differences were found between both stations. Once again, if the same regulated values are considered, all the water samples exceeded largely the value legislated. As for the sediment samples, all the samples were contaminated with Pb. The BSB station seemed to be more polluted than the ML station, but no significant differences were found by the Two-Way ANOVA analysis. However, this fact was expected once the BSB station was more influenced by freshwater uptakes, by runoff and by the higher residence time of water than on the ML station.

At last, almost all the cockle samples were contaminated with Pb on both sampling stations. At the ML station, the degree of Pb contamination in the animals' tissues seemed to be highest, but no significant difference was found between stations. The value fixed by the European Commission Regulation (EC) No 1881/2006 for maximum levels of Pb on bivalve molluscs is 1.5 mg.kg^{-1}(w.w.), but comparing all the values detected on the *C. edule* samples at both stations, it is possible to infer that all the values detected, except on spring 10 at the ML station, are above the legal value.

On rivers and surface water, nickel is present in an ionic form and in stable organic complexes, which become absorbed on bottom sediments (Von Burg, 1997). Regarding the environmental samples, only the results detected on the dissolved fraction of the water samples were relevant. Contamination by Ni on these samples was more frequent at the ML station, as compared to the BSB station. The highest value was detected on both stations on summer 10 (± 0.029 mg.l^{-1}). This season was in fact identified as being significantly different from the other seasons (Two-Way ANOVA procedures). The Directive 2008/105/CE fixed the value of contamination on interior surface waters at the maximum value of 0.02 mg.l^{-1} (**Table 2.1)**. So, comparing both values, contamination with Ni on summer 10 was above the legal value allowed. As regards to the biological samples, all the samples analysed were contaminated with Ni. The concentrations detected in the bivalves from the BSB station were statistically and significantly higher than at the ML station, proving that the bivalves from the BSB station accumulated in their tissues higher concentrations of Ni during the study period.

As Williams & Hudson (2014) referred, biomonitoring studies are frequently applied to assess the impact of pollution on a marine ecosystem. Furthermore, Duarte *et al.* (2011) referred that the molluscs have the ability to accumulate metals from their food and surrounding seawater in concentrations that exceed considerably those found in their natural environment. Therefore, physiological changes of these organisms can be related with the effects of toxic chemicals present in water and that were accumulated in their tissues. Physiological responses have also been used as early indicators of potential ecosystem damage caused by metals and organic contaminants and can offer a clear indication of ecosystem health (Nilin *et al.*¸ 2012; Williams & Hudson, 2014). For all these reasons, in the present study a biometric characterization (length, height and dry weight) of *C. edule*, and calculations of both the bioaccumulation factor (BAF) and the concentration factor (CF) for each metal were performed, in order to determine and to better understand the effects of the pollution by Cd, Pb and Ni on the cockles of the Óbidos Lagoon.

For all the biometric variables analysed in the present study, an interaction between the factors sampling station and sampling season was detected by Two-Way ANOVA techniques. On the ML station, the cockles had significantly higher lengths and dry weights on summer 10 when compared to autumn 09 and winter 09. On the BSB station, the cockles presented significantly distinct lengths, heights and dry weights on spring 09 and autumn 09 when compared with summer 09 and summer 10. These results can be explained by the variation on the physicochemical parameters observed on the seasons and stations during this study. Some references support these results, since the growth of *C. edule* is known to be influenced by the age, the season, the geographical location, the tidal height, the water temperature, the food availability, the population density and, also, the interspecific competition (Crespo *et al.*, 2010; Dabouineau & Ponsero, 2011). Recent studies by Verdelhos *et al.*, (2015a, b) have characterized the *C. edule* as a tolerant organism to a wide interval of temperatures and salinities (euryhaline). They defined too, an optimum range of temperature and salinity for the cockle *C. edule* normal activity, namely 20-23ºC and 20-25‰ and critical values (death of the cockle) at T > 32ºC and S <10‰. All of these data demonstrate that *C. edule* is a sensitive indicator of the environmental fluctuations.

To better evaluate the bioaccumulation abilities of *C. edule* in the presence of environmental contaminants, calculus of BAF and CF were performed whenever the metals were detected simultaneously on the environment (sediment or seawater, respectively) and on the animals' tissues.

Apparently, the cockles at the ML station (BAF_{min} = 0.0005; BAF_{max} = 0.384) were more willing to accumulate Pb on their tissues than the cockles from the BSB station (BAF_{min} = 0.018; BAF_{max} = 0.024). Moreover, it is possible to observe that, on winter 10, the BAF value obtained at the ML station was significantly distinct from the other seasons. Comparing this conclusion with the metal contamination of the sediments, this result was curious. In most seasons the metal contamination on the sediment was higher at the BSB station. Another curious fact was that, despite the higher degree of bioaccumulation observed in the cockles (BAF = 0.384) in winter 10, the contamination of the sediments in this specific season was the lowest observed during the study period. Thus, the contamination with Pb on cockles seems not to come exclusively by the accumulation of Pb from the sediments. A similar conclusion was reported by Velez *et al.* (2015) with the clams (*Venerupis corrugata, Ruditapes decussatus and Ruditapes philippinarum*) on *Ria de Aveiro*. In that study, clams from the less contaminated areas tended to have higher BAF values than the clams from the most contaminated ones. Also, it is important to refer that, like as observed by Velez *et al.* (2015), in our study Pb was the most abundant element in the sediments and the BAF values estimated were ≤ 0.384. This may mean that the cockles at the Óbidos Lagoon, like the clams on *Ria de Aveiro*, do not bioaccumulate this metal in their tissues from the metal present on the sediments. However, it is important to refer that the BAF on winter 10 was much higher from the other values obtained. Probably, the specific environmental fluctuations that characterized this season at the Óbidos Lagoon, such as the high precipitation and the artificial interventions to relocate the “Aberta”, affected the bioaccumulation ability of Pb on this organism.

As previously explained in the Results section, the CF assessment was only performed for the metals Pb and Ni. For the CF Pb, it is possible to observe that the cockles at the ML station concentrated Pb more regularly during the seasons analysed in the present study than the cockles at the BSB station. Two CF Pb values were significantly higher than the others, so the cockles have accumulated more Pb on their tissues on that seasons. One value occurred at the ML station on winter 10 ($2.48 \times 10^{+3} \pm 5.4 \times 10^{+2}$ $l.kg^{-1}$) and another at the BSB station on summer 10 ($4.22 \times 10^{+3} \pm 2.6 \times 10^{+3}$ $l.kg^{-1}$).

For the CF of Ni contamination, and as observed for CF Pb, the cockles at the ML station concentrated Ni more regularly, as opposed to the cockles from the BSB station. Statistically, it was shown that seasons influenced the ability of cockles to concentrate this metal on their tissues, especially on spring 09 ($2.49 \times 10^{+3} \pm 3.4 \times 10^{+2}$ $l.kg^{-1}$) as opposed to summer 10 ($5.07 \times 10^{+2} \pm 3.4 \times 10^{+2}$ $l.kg^{-1}$) and winter 09 ($1.89 \times 10^{+2} \pm 9.2 \times 10^{+1}$ $l.kg^{-1}$). Combining this result with the biometric variables on the same seasons, especially dry weight and length, the data seems to suggest that on spring 09 the cockles at ML were smaller than on the other seasons, so these organisms were more sensitive to the presence of $Ni_{dissolved}$, concentrating this metal more efficiently.

Comparing the CF values for Pb and Ni with the study performed by Conti *et al.* (2011) on *Mytilus chilensis*, it can be possible to suggest that *C. edule* seems to have more ability to concentrate Pb and less with Ni on their tissues than the mussel (CF Pb = $0.37 \times 10^{+3}$ $l.kg^{-1}$ and CF Ni = $9.5\ 10^{+3}$ $l.kg^{-1}$). However, CF results must be analysed carefully, because this parameter exhibits huge temporal and spatial variations (Sousa *et al.,* 2009).

The cockle *C. edule* is commonly captured on the Óbidos Lagoon for Human consumption by the local population but also commercially. Therefore, it was important to assess public health risks about the different metal concentrations determined during the present work. The dietary assessment allows assessing the quantity of bivalves that an adult with 70kg can consume per week so as not to exceed the Provisional Tolerable Weekly Intake (PTWI) fixed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Previously on this Discussion section, it was compared the contamination values detected on cockle’s samples with the maximal’s values on wet weight fixed by EFSA.

As explained on the Results section, the metal concentrations detected on the cockles were expressed on dry weight. So, to compare the data with the regulated values the ratio wet/dry weight of the soft tissues on the biological samples studied was estimated to be10x. Considering this analyses, it was concluded that Cd and Pb were the major concern.

The presence of Cd on the Óbidos Lagoon was not detected by the analytical equipment during almost the entire study period. However, for the unique value detected on one cockle sample from the ML station, it can be possible to infer that, if similarly contaminated cockles were consumed, it would be necessary to consume 0.459kg (w.w.) of cockles per week to exceed the PTWI value for an adult of 70kg. It's a low quantity of cockles per week, so it was possible to infer a certain degree of risk for the Human health with the cockle consumption. However, it was reported on literature that *C. edule* has a high propensity to accumulate this toxic metal, and thus maximum levels in mollusc can easily be reached, even in areas with low Cd contamination (Figueira *et al.*, 2011; Velez *et al.*, 2015). So, it is necessary to maintain the biomonitoring of this metal to rapidly identify this type of contamination for the environment and Human health.

Pb contamination shows another reality, since the PTWI for an adult of 70kg was fixed on 1.75mg.kg^{-1}.week^{-1}. On the higher contamination scenarios (ML station = 44.851 mg.kg^{-1} (d.w.); BSB station = 13.136 mg.kg^{-1} (d.w.)), it is only necessary to consume a few grams of cockles per week to reach the PTWI value, at both stations. On the other hand, considering the minimum Pb contamination values observed (ML station), a consumption of more than 1kg.week^{-1} (w.w.) of cockles is allowed. These values are worrying for the Human health regarding the consumption of this cockle species, so special attention is necessary for this metal and regular biomonitoring programs should be encouraged.

The PTWI for the element Ni is higher than for the two other metals and is fixed on 2.45 mg.kg^{-1}.week^{-1}. To attain this value, on the maximum contamination scenarios (ML station = 7.013 mg.kg^{-1} (d.w.); BSB station =15.424 mg.kg^{-1} (d.w.)) it would be necessary to ingest only a few grams of C*. edule* from both stations. On the minimal contamination, a consumption of more than 300 g of cockles per week is possible. Although this metal is referenced as non-toxic to humans at slight quantities (Conti *et al.*, 2011), this value of consumption is very low. Thus, a high consumption of cockles with this metal concentration can become worrying for Humans health, so for this metal it is recommended increased monitoring too.

Although the results above mentioned were not excellent, if the seasons on which these contaminations were detected are taken into account, it is possible to infer that the season with the maximum contaminations on the ML station corresponds with a period of low shellfish harvesting (winters). For the BSB station the maximum (summer 10) and minimum (Pb - spring 09; Ni - autumn 09) values were detected on seasons with higher harvesting activities, but on this sampling station shellfish harvesting is not a common activity. On the other hand, the detection of the metal concentrations on cockles was also important to understand the impact of metal contamination on the ecosystem, especially on the trophic chains. It is important to remember that *C. edule* belongs to one of the most important macrofaunal groups on estuaries – bivalves. This group is essential to the community energy flow, contributes to water column purification and is an important power supply for some other species existing on the lagoon (Verdelhos *et al.*, 2015). So, if this cockles bioaccumulated metals, it is possible to transfer this contamination to the higher trophic levels.

On the present work, the influence of the presence of contaminants on the biometric parameters, BAF and CF of the cockles' samples were studied. Some correlations were determined, some more relevant than the others.

The strong positive correlation between [Pb] and BAF Pb and CF Pb were already expected whereas the factors are calculated with Pb contaminations values. So, the contaminations with Pb on the tissues of cockles have a correlation with the capacity to bioaccumulate the metals from the environment.

The positive and modest relationship between CF Ni and Ni was lower than the expected. Probably, the accumulation of Ni on *C. edule*'s tissues was not only related to the availability of this metal on the dissolved fraction of the water. However, the modest correlation observed between CF Ni and [Pb], appears to suggest that, a relationship, between the accumulation of Pb and Ni on cockles when these two metals are present on the environment, may exist.

The positive and modest correlation with length and height were expected too, but not the low value. Probably, it is due to the dimension of the samples, N = 6 for each station and on each season, however it was possible to conclude that when the cockles grow on length, there is a growth on height too.

The positive and modest correlation between length and dry weight may be due to the t fact that the weights of the cockles depend on various environmental factors, namely, temperature, daily food availability and intake, season, location. For example, the same cockles on winter and on spring have a higher weight on spring (sexual maturation period) than on winter (low food availability).

The dry weight of the animals and [Ni] were negatively correlated in a modest relationship. This result seems to indicate that lighter cockles accumulated more Ni or that a higher degree of Ni contamination on cockles, lowers the dry weight of the organisms. A similar relationship was observed with CF Ni.

The positive and modest correlation among shells height, [Pb] and consequently BAF Pb and CF Pb shows that cockles with a higher height concentrated more Pb or maybe the Pb accumulation can affect the growth of the cockles causing an increase of the height instead of the length.

Finally, the modest and positive correlation between $[Ni]_{dissolved}$ and $[Pb]_{suspended}$ on water samples shows that exists a relationship on the availability of Ni dissolved in the presence of Pb particulated. This relationship was reflected too on the following correlations obtained before on cockles: CF Ni - [Pb] and CF Ni – [Ni]. These results seem to demonstrate that the Ni dissolved was less concentrated by the cockles on the presence of Pb particulated.

5. CONCLUSION

5. Conclusion

The present study allowed concluding that during the 7 periods, the water temperature and salinity were different between the ML station and BSB station demonstrating clear seasonal fluctuations of these parameters.

Metal contamination with cadmium was sporadically detected on water fractions and biological samples but when present, the concentrations obtained were above the threshold fixed by the legal authorities. Once again, the occurrence of this metal on suspended water fraction was related to seasonal fluctuations (precipitation winter 10).

Metal contamination with lead was present on all the environmental and biological samples and normally these were above the values regulated. The occurrence of this metal on suspended water fraction was linked with seasonal fluctuations (precipitation).

Finally, metal contamination with nickel was detected on water fractions and on biological samples, but not on the sediments, and it can be considered that the values obtained were close to the values legislated. This metal shows seasonal fluctuations too (summer 10) on water dissolved fraction.

The physiological responses of *C. edule* show a spatial and seasonal fluctuation during the study period. Moreover, *C. edule* is a sensitive indicator of the environmental fluctuations showing bioaccumulation behaviour for metal Pb and preferentially for metal Ni. The results suggest that Pb contamination on the cockles does not come preferentially by the metal contamination from the sediments or water dissolved fraction.

Concerning, the assessment of PTWI, the cockles at BSB station have shown to be inappropriate to consumption for both metals (Pb and Ni), independently of the season. On ML station, it was shown that winter 10 was the worst season to consume cockles while spring 10 was better for Pb and Ni contaminations. However, these results are not alarming since shellfish harvesting is not practiced at the BSB station, or on winter seasons, although regular monitoring studies targeted to these two specific metals are highly recommended.

As a whole, on this study, *Cerastoderma edule* revealed to be a good sensitive indicator of the wide fluctuations that occurred on the Óbidos Lagoon, namely with the physicochemical conditions. Regarding, the metal contamination, this organism was clearly affected by the wide disturbances occurring on their environment, as demonstrated by the winter 10 on the present study, especially for the metals Pb and Ni that were, between the three metals analysed, the prevailing on the ecosystem during the study period.

6. FUTURE PERSPECTIVES

6. Future Perspectives

In the course of this study, the common cockle *Cerastoderma edule* has revealed to be a sensitive indicator of wide spatial and environmental fluctuations, such as the ones referred on some studies. But to better understand the effects of the metal contamination on the cockle, further studies could be realized:

- ✓ Calculus of the Condition Index (CI) on fresh cockles, without depuration, could be studied to give an indication of the general physiological status of the animals when they are harvested.
- ✓ Analyse the relationship between the metal contamination on cockles with the metal contamination on water suspended fraction, and developing a calculus of bioaccumulation similar to BAF and CF based on suspended metals. This approach would allow to understand if the Pb contaminations on cockles were related with the Pb water suspended fraction contaminations.
- ✓ Investigate to a deeper level the correlation between Ni dissolved and Pb suspended on water samples, and if this relationship has repercussions on the bioaccumulation ability of *C. edule*.
- ✓ Monitoring the accumulation of metals Cd, Pb and Ni at laboratory controlled conditions to better understand the relationships between these metals and *C. edule.*
- ✓ Identify the biological mechanism triggered when the cockles are in the presence of metals.
- ✓ Study the bioaccumulation behaviour of common cockle to other contaminants as copper (Cu), mercury (Hg), iron (Fe) and manganese (Mn) at the Óbidos Lagoon on the same stations.

Considering these approaches, there is a wide range of potential studies that could allow a better understanding of the close relationship between the metal contaminations on the environment and on the cockles, so that the use of *Cerastoderma edule* as a regular bioindicator in environmental monitoring studies could be improved.

7. REFERENCES

7. References

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