POLITÉCNICO DE LEIRIA
ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Assessment of trace metals contamination (Cu, Fe, Mn, Zn, Cd and Pb) at the Óbidos Lagoon using Hediste diversicolor *(O. F. Müller, 1776) as a bioindicator*

Catarina Sequeira Bruno

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Dissertation submitted to obtain the degree of Master in Biotechnology of Marine Resources

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“Gazing through the window at the world outside
Wondering will mother earth survive
Hoping that mankind will stop abusing her
Sometime
(…)
Watching all of history repeat itself
Time after time

I'm just a dreamer
I dream my life away
I'm just a dreamer
Who dreams of better days (…)”

Dreamer – Ozzy Osbourne

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Resumo

Os ambientes estuarinos representam um grande potencial ecológico e económico, e consequentemente são amplamente explorados (por exemplo, a Lagoa de Óbidos). As atividades antropogénicas são a principal fonte de contaminação por metais traço, elementos que se acumulam no ambiente e nos organismos.

A espécie de poliqueta *Hediste diversicolor*, presente na Lagoa de Óbidos, preenche todos os requisitos de um bioindicador. Esta espécie foi considerada tolerante a poluentes por diferentes autores, em ambientes estuarinos distintos. Como tal, no presente estudo, o principal objetivo foi utilizar *H. diversicolor* como bioindicador de contaminação por metais traço na Lagoa de Óbidos, avaliando a acumulação de Cu, Fe, Mn, Zn, Cd e Pb, sazonalmente, durante o período de um ano (de 2018 a 2019) em quatro estações de amostragem diferentes (BB, AR, CM e PF). Os resultados foram complementados com a análise da contaminação por metais em amostras de água e sedimentos. Foram também estudadas algumas respostas fisiológicas das poliquetas, a fim de melhor compreender os efeitos da presença de metais e da sua acumulação nestes animais.

Todos os metais estudados foram detetados na água, nos sedimentos e nas amostras biológicas. Os metais essenciais Fe e Zn foram detetados em concentrações mais elevadas em todas as amostras biológicas, contrariamente ao Cu e ao Mn. No geral, os organismos provenientes da Lagoa de Óbidos aparentam ser menos tolerantes ao Zn comparando com os restantes metais essencias. Os resultados sugerem que o elemento Mn está claramente relacionado com o crescimento dos organismos, e ainda que o Cu pode ter também influência no crescimento dos organismos.

Relativamente aos metais não essenciais, o elemento Cd está menos presente em ambas os tipos de amostras, ambientais e biológicas. O metal Pb aparece em concentrações mais elevadas nas poliquetas quando comparado com o Cd e os resultados indicam que os organismos são mais tolerantes ao Cd do que ao Pb, o qual aparentemente afeta de forma negativa o crescimento dos organismos. Os metais detetados no ambiente e as respostas fisiológicas das poliquetas a estes elementos sugerem uma maior contaminação no Braço da Barrosa (BB), seguida pela estação dos rios Arnóia e Real (AR).

Este estudo permitiu concluir que a poliqueta *Hediste diversicolor* é um bioindicador sensível de contaminação por metais traço na Lagoa de Óbidos.Com efeito, a preocupação com a contaminação de metais neste ambiente estuarino continua a ser uma questão importante para o futuro deste ecossistema. Deste modo, a contínua monitorização da contaminação dos estuários pode assegurar o futuro de muitas espécies, incluindo o ser humano, que é dependente dos estuários para múltiplas atividades, mas também é a maior fonte de impacto e contaminação destes ambientes.

Palavras-chave: *Hediste diversicolor*; Metais traço; Contaminação; AAS; Lagoa de Óbidos; Capacidade de acumulação

Abstract

Estuarine environments hold a large ecologic and economic potential, and consequently they have been extensively exploited (*e.g.* Óbidos Lagoon). Pollution from anthropogenic activities is the main source of contamination of trace metals and these elements accumulate in the environment and in the organisms.

In estuaries, invertebrates such as polychaetes, commonly used as bait, for fishing, could also be an efficient bioindicator of contaminants, such as trace metals. The species of ragworm *Hediste diversicolor* found at Óbidos Lagoon meets all the requirements as a bioindicator. This polychaete was reported as tolerant to pollutants by several authors in different estuarine environments. As such, on the present study, the main objective was to use *H. diversicolor* as a bioindicator for contamination by trace metals at the Óbidos Lagoon, by evaluating the accumulation of Copper, Iron, Manganese, Zinc, Cadmium and Lead, on a seasonal basis, during the period of one year (2018 to 2019), at four different sampling stations (BB, AR, CM and PF). The results were complemented by the analyses of metal contamination in water and sediment samples. Some physiological responses of these ragworms were also studied in order to better understand the effects of the metals' presence and their accumulation by these animals.

All the studied metals were detected in water, sediments and biological samples. The essential metals Fe and Zn were detected in higher concentrations in all biological samples, contrarily to Cu and Mn. In general, the organisms from Óbidos Lagoon appeared to be less tolerant to Zn when compared with the other essential metals. The element manganese was clearly related with organisms' growth and the results suggest that Cu may also have influence on the organisms' growth.

Regarding non-essential metals, the element Cd was less frequent in both environmental and biological samples as compared to Pb. Also, the metal Pb appears in higher concentrations in the ragworms when compared with Cd. The results suggest that *H. diversicolor* is more tolerant to Cd than Pb, which appears to affect negatively the organism's growth.

Globally, the metals detected in the environmental samples and the physiological responses of the ragworms to these elements suggest higher contamination at *Barrosa*'s Branch, followed by *Arnóia and Real* rivers. This study allowed to conclude that *Hediste* *diversicolor* was a sensitive bioindicator of trace metals contamination at Óbidos Lagoon. Also, contamination by metals in Óbidos Lagoon remains an issue of concern and will have a determinant influence on the future of this ecosystem. Therefore, the continued monitoring of estuaries contamination may ensure the future of multiple species, including humans, which are dependent on estuaries for multiple activities but are also the main source of contamination on these environments.

Key-words: *Hediste diversicolor;* Trace metals; Contamination AAS; Óbidos Lagoon; Bioaccumulation ability;

Table of contents

List of Figures

List of Tables

List of Abbreviations & Acronyms

°C	Degree centigrade
%	Percentage
$[Cd]_{Dis}$	Cadmium concentrations dissolved in the water column
$[Fe]_{Dis}$	Iron concentrations dissolved in the water column
$[Mn]_{Dis}$	Manganese concentrations dissolved in the water column
$[Cd]_{Org}$	Cadmium concentrations in the organisms
$[Cu]_{Org}$	Copper concentrations in the organisms
$[Fe]_{Org}$	Iron concentrations in the organisms
$[Mn]_{Org}$	Manganese concentrations in the organisms
$[Pb]_{Org}$	Lead concentrations in the organisms
$[Zn]_{Org}$	Zinc concentrations in the organisms
$[Cd]_{Sed}$	Cadmium concentrations in the sediment
$[Cu]_{Sed}$	Copper concentrations in the sediment
$[Fe]_{Sed}$	Iron concentrations in the sediment
$[Mn]_{Sed}$	Manganese concentrations in the sediment
$[Pb]_{Sed}$	Lead concentrations in the sediment
$[Zn]_{Sed}$	Zinc concentrations in the sediment
$[Cu]_{Sus}$	Copper concentrations suspended in the water column
$[Fe]_{Sus}$	Iron concentrations suspended in the water column
$[Mn]_{Sus}$	Manganese concentrations suspended in the water column
$[Zn]_{Sus}$	Zinc concentrations suspended in the water column
$[Cd]_{Sus}$	Cadmium concentrations suspended in the water column
$[Pb]_{Sus}$	Lead concentrations suspended in the water column
AAS	Atomic Absorption Spectrometry
AR	*Arnóia* and *Real* Rivers
BAF	Bioaccumulation Factor
BB	*Barrosa*’s Branch
BOD	Biochemical Oxygen Demand
BS	Body Size
BSB	*Bom Sucesso* Branch
Cd	Cadmium
Cd_{BAF}	Cadmium bioaccumulation factor in *H. diversicolor*
Cd_{CF}	Cadmium concentration factor in *H. diversicolor*
$Cd_{Sediment}$	Cadmium concentration in the sediment
CF	Concentration Factor
$CHCl_3$	Chloroform
CH_3OH	Methanol
cm	Centimetre(s)

CM	*Covão dos Musaranhos*
Co	Cobalt
COD	Chemical Oxygen Demand
C_s	Metal concentration in sediment
Cr	Chromium
Cu	Copper
$Cu(NO_3)_2$	Copper nitrate
Cu_{BAF}	Copper bioaccumulation factor in *H. diversicolor*
Cu_{CF}	Copper concentration factor in *H. diversicolor*
C_w	Metal concentration in water column
df	Degrees of freedom
EFSA	European Food Safety Authority
EQS-AM	Environmental Quality Standard-Arithmetic Mean
F	Female organisms
FAAS	Flame Atomic Absorption Spectrometry
Fe	Iron
Fe_{BAF}	Iron bioaccumulation factor in *H. diversicolor*
Fe_{CF}	Iron concentration factor in *H. diversicolor*
$Fe(NO_3)_2$	Iron nitrate
g	Gram(s)
$g.L^{-1}$	Gram(s) per litre
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
h	Hour(s)
H. diversicolor	Polychaetes *Hediste diversicolor*
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
IARC	International Agency for Research on Cancer
IST/IPIMAR	*Instituto Superior Técnico/Instituto Português de Investigação das Pescas e do Mar*
kg	Kilogram(s)
km^2	Kilometre(s) per square
L	Litre(s)
L3	The first three segments of ragworms (peristomium, prostomium and first chaetiger)
$L.kg^{-1}$	Litre(s) per kilogram
$L.\mu g^{-1}$	Litre(s) per microgram
m	Metre(s)
$m^3.s^{-1}$	Cubic metre(s) per second
mg	Milligram(s)
$mg.L^{-1}$	Milligram(s) per litre
$mg.kg^{-1}$	Milligram(s) per kilogram
mg/m^3	Milligram(s) per cubic metre

$Mg(NO_3)_2$	Magnesium nitrate
mL	Millilitre(s)
mm	Millimetre(s)
Mn	Manganese
Mn_{BAF}	Manganese bioaccumulation factor in *H. diversicolor*
Mn_{CF}	Manganese concentration factor in *H. diversicolor*
$Mn(NO_3)_2$	Manganese nitrate
$mol.L^{-1}$	Moles per litre
N	North
na	Not applicable
ND	Non determinate
Ni	Nickel
nM	Nanomol(es)
OMC	Organic Mater Content
Pb	Lead
Pb_{BAF}	Lead bioaccumulation factor in *H. diversicolor*
Pb_{CF}	Lead concentration factor in *H. diversicolor*
PET	Polyethylene Terephthalate
PF	*Poça das Ferrarias*
ROS	Reactive Oxygen Opecies
SNIRH	Sistema Nacional de Informação de Recursos Hídricos
T	Temperature
W	West
WW	Wet Weight
WHO	World Health Organization
WWTP	Waste Water Treatment Plant
Zn	Zinc
Zn_{BAF}	Zinc bioaccumulation factor in *H. diversicolor*
Zn_{CF}	Zinc concentration factor in *H. diversicolor*
$Zn(NO_3)_2$	Zinc nitrate
$\mu g.g^{-1}$	Microgram(s) per gram
$\mu g.L^{-1}$	Microgram(s) per litre
µm	Micrometre(s)
µM	Micromol(es)
ρ-spearman	Spearman correlation

1. Introduction

1.1 Pollution and Estuarine Environments

Environmental pollution is a problem of great concern and has recently gained much attention. The growth of human populations and the continuous release of prejudicial substances into the environment by humans cause its deterioration and decline (Eisler, 2007). Coastal and marine environments are the most impacted areas by contamination and human pressure (Lotze *et al*., 2006), especially because most human populations tend to settle in coastal areas. Estuarine environments, in particular, are a relevant and substantial example, since these habitats have been places of intense human occupation (Lotze *et al*., 2006). As a consequence, these ecosystems are also considered the most impacted from pollution by environmental contaminants resulting from anthropogenic activities, alterations due to global change and also to invasion by exotic invasive species (Elliott *et al.,* 2014) leading to negative impacts on these environments and, ultimately, on human health. On the other hand, economic activities, like agriculture and fishing, may also be influenced (Amiard-Triquet & Rainbow, 2009).

Estuaries are one of the most important and productive natural environments, as they provide habitat and safety for different organisms, including a high diversity of animal and plant species (Ribeiro *et al*., 2018). They are characterized by a high biological productivity, serving as nursery areas for several species (Kennish, 2002) and also provide habitat to organisms for the whole or part of their life cycle (Jordan, 2012).

After the implementation of the Water Framework Directive (Directive 2013/39/UE), the European Union member states were re-incited to monitor the ecological quality of all their water bodies, with focus on the coastal environments, due to their ecological vulnerability. Estuarine environments are characterized by their complexity. River freshwater mixes with sea water from the open ocean (Cochran, 2014). The river flow, the tides (Lara *et al*., 2019) and the winds (Wang *et al*., 2017), are important factors, which lead to a complex environment, where temperature, salinity and organic matter continuously fluctuate (Cochran, 2014). Freshwater discharge could be from many different sources. Natural water fluxes may come from sea, rivers and atmosphere. However, several water fluxes from anthropogenic sources are usually disposed in these ecosystems through rivers, and are mostly from urban runoff, industrial and rural activities, agricultural effluents, domestic discharges, construction waste and sewage wastewater (Amiard-Triquet & Rainbow, 2009).

Coastal lagoons are estuarine environments with little freshwater influence, where the shallow water systems are connected to the sea by one or more entrances (Oliveira *et al.*, 2006). These characteristics create favorable conditions for the accumulation of sediments, since the deficient water renewal may slow down the dilution process and increase sediment deposition and contaminant retention (Mucha *et al.*, 2004). The estuarine sediments differ from sand to mud and silt-clay, are rich in toxic agents (Bouraoui *et al.*, 2016) and could be considered reservoirs of the trace elements that do not remain soluble in water. Consequently, sediments can be used as indicators of local pollution conditions (Ribeiro *et al.*, 2018).

1.2 Bioaccumulation of metals and its impacts in the ecosystem

Metals such as Cd and Pb are a serious environmental problem since they are highly toxic, even in trace amounts. Cu, Fe, Zn and Mn are essential however in excess could also be toxic. The continuous contamination by metals and the lack of their remediation leads to their persistence and bioaccumulation in food chains, increasing the risks for human health. The environment and the organisms' health is affected, from plants to animals, which leads to the ecosystem degradation (Amiard-Triquet & Rainbow, 2009; Kabata-Pendias, 2011). Despite the fact that metals occur naturally in the environment, they also enter through anthropogenic processes (Song *et al.*, 2014). This pollution comes from industrial waste, fossil fuel combustion, sewage wastewater, energy production and construction (Kabata-Pendias, 2011), increased mining and beach nourishment activities which are associated to rapid urbanization, intensive economic development and continuous population growth (Wang *et al.*, 2017). These elements are one kind of pollutants discharged to estuarine environments by the river basins (Veiga *et al.*, 2019). These contaminants can occur in different forms and exhibit different physical and chemical behaviors. Trace metals can stay dissolved in the water and may be removed from water to sediment via sedimentation or be ingested by organisms (Kabata-Pendias, 2011). Metal contamination and accumulation in water are related with the continuous change in physicochemical factors such as, temperature, dissolved solids, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) (Phiri *et al.*, 2005). Redox potential and pH are also important factors (Pedro *et al.*, 2013) . Generally, if pH is low, some metals toxicity may increase (Ribeiro *et al.*, 2018). Beside their toxic behavior, metals have a long range transport via suspended particles, also an accumulative effect in the sediment and can easily enter in the food chains. Consequently, the principal reservoirs of metals are water, sediment and biota (Amiard-Triquet *et al.*, 2015) and analyzing the distribution of metals in sediment, water and biota is useful to trace the degree of contamination, mostly induced by anthropogenic pressures (Conceição *et al*., 2013).

Several elements occur in trace amounts in living organisms and some of them are essential for their survival. On the other hand, there are non essential elements which are not necessary to any biological function and also can lead to organisms stress and death. Essential metals are involved in the growth, development and health of the organisms. However, quantitative difference between essential amounts and a biological excess, usually is minimal. Non essential metals are not involved in organisms development on the contrary, these elements could affect organisms growth and, in certain conditions, lead to their death (Eisler, 2007).

Essential metals

Copper – Cu

Copper is an essential trace metal found in food, air, water (WHO, 1996) and soils. This element occurs in the Earth's crust at an average of 55 mg.kg^{-1} and shows tendency to concentration in mafic rocks and argillaceous sediments (Kabata-Pendias, 2011).

Copper pollution is associated with natural sources but, in particular, with human activities, such as effluents discharge from agriculture, livestock production, ore mining and the manufacture of medicines (WHO, 2007). Also it is used to manufacture electrical equipment (Eisler, 2007), water pipes, roof coverings, pesticides and fertilizers, inorganic dyes and feed additives (WHO, 2007).

This trace metal is essential for the normal growth and metabolism of all living organisms (Eisler, 2007), from yeasts to mammals. They depend upon copper, which guarantees the correct function of several copper-binding proteins. This element acts both as a cofactor and as an allosteric component of several enzymes, such as cytochrome-c oxidase and superoxide dismutase (Uauy *et al*., 1998).

Humans ingest copper manly via food and drinking-water (WHO, 1996). Low levels of copper might induce nutrient deficiency and highest levels could be acutely toxic (Eisler, 2007). At concentrations above approximately 3 mg.L^{-1}, copper has been shown to cause acute gastrointestinal discomfort, while nausea may occur at concentrations of 3 mg.L^{-1} (WHO, 2007). The lethal oral dose for adults may be observed between 50 and 500 mg of copper(II) salt per kg of body weight (WHO, 1996).

Iron – Fe

Iron is the most important metal, its average content of the Earth's crust is about 5% and it is not considered a trace element in rocks and soils (Kabata-Pendias, 2011). In nature, it is mostly found in the form of its oxides and occurs in air, water, food and soil (WHO, 1996).

Iron can be found in all living organisms. This metal plays an important role in the behaviour of other trace minerals and in many biochemical reactions, such as element of several enzymes and part of oxygen transport, by bounding to haemoglobin that is an essential blood protein (McDowell, 2003). In addition, this ellement plays an important role in the tissues of the gastrointestinal tract (EFSA, 2006), which makes iron an essential element for humans, and for other multiple animals, such as mice, ruminants, and fish (McDowell, 2003). Also, it is an indispensable nutrient in plants and in microorganisms (Kabata-Pendias, 2011).

As an essential metal, the insufficient ingestion of iron is associated to several health problems in humans and animals, such as anaemia (McDowell, 2003). However, ingested in levels between 50 to 220 mg Fe.day^{-1} side effects include nausea, vomiting, heartburn, epigastric discomfort, diarrhea and intractable constipation. Also, an oral dose of 60 mg Fe.kg^{-1} body weight can be lethal (EFSA, 2006).

Manganese – Mn

Manganese is one of the most abundant trace metals in the lithosphere (WHO, 1996; Kabata-Pendias, 2011). It commonly appears together with iron (WHO, 1996) and there are several of Mn minerals, mainly associated with other metals (Kabata-Pendias, 2011). This element can be found in air, water, human food and soils (WHO, 1996). Manganese is used to provide hardness and toughness, and as antioxidant in steel and alloys production. Also it is applied for the production of pigments, ceramics, glass and used as fertilizer and as livestock supplement (Kabata-Pendias, 2011).

Manganese is an essential trace metal for several species, since microorganisms to plants and humans (Kabata-Pendias, 2011). It is a component of the enzymes arginase, pyruvate carboxylase and superoxide dismutase, and acts as co-factor of certain enzyme systems. A deficiency intake of this metal can affect both the metabolism and growth of the organisms (EFSA, 2006). Humans ingest manganese mainly from food. However, when inhaled and exposed chronically, the neurological effects in humans are weakness, anorexia, muscle pain, apathy, slow speech, monotonous tone of voice, known as "manganism" syndrome. Also, the minimal exposure level producing neurological effects is not certain, but is believed to be between 0.1 and 1 $mg.m^{-3}$ (WHO, 1996).

Zinc – Zn

Zinc is an essential trace element for all living organisms. This metal is a constituent of more than 200 metalloenzymes and other metabolic compounds. Also, it is responsible for stability of biological molecules and structures, such as DNA, membranes and ribosomes (Eisler, 2007). This element can be found in air, water, human food and soils (WHO, 1996). Industrially, zinc is used as a catalyst in the production of different chemical substances such as rubber, pigments and pesticides (Kabata-Pendias, 2011). However, this trace metal is mainly used to the production of noncorrosive alloys, brass, galvanizing steel and iron products (Eisler, 2007) to apply in batteries, automobile equipment, pipes and household devices (Kabata-Pendias, 2011).

As an essential metal, its deficiency causes health problems. However, in larger amounts it can also be toxic. The consumption of more than 500 mg of zinc sulfate could origin fever, nausea, vomiting, stomach cramps and diarrhea (WHO, 1996).

Nonessential metals

Cadmium – Cd

Cadmium is a nonessential and toxic trace metal, which rarely occurs in nature in its pure form. The average Cd content for the Earth crust is given as 0.1 $mg.kg^{-1}$

(Kabata-Pendias, 2011). Naturally, it is present with zinc and lead in sulphide ores (WHO, 1996) and occurs in the environment in its inorganic form, as a result of volcanic emissions and weathering of rocks. Additionally, human activities also have a great impact in Cd levels (Kabata-Pendias, 2011). As a result, cadmium is present in the air, soils, food and water. For unpolluted water cadmium concentrations are below 0.1 $\mu g.L^{-1}$ (WHO, 1996).

Cadmium is obtained as an industrial by-product of the production of zinc, copper and lead. This trace metal is used in pigment production and in the manufacture of plastic stabilizers and batteries (Eisler, 2007). Smelter fumes and dusts from waste and fossil fuels incineration, wastewater and fertilizers are also sources of cadmium contamination (Eisler, 2007; EFSA, 2012).

Considering its large solubility in water (Pedro *et al*., 2013) and its transference to soil by wet or dry deposition, this trace metal can easily enter in the food chains (WHO, 2000). It is believed that populations are exposed to cadmium mainly from cigarette smoking, food (WHO, 2000) and water (EFSA, 2012).

There are no evidences of cadmium being essential or benign to organisms. In fact, it has been involved as the cause of numerous human deaths, and several deleterious effects in fish (Eisler, 2007). Moreover, in sufficient concentrations, this trace metal is toxic to all forms of life since microorganisms to higher plants, animals and humans (Eisler, 2007). Continuous exposures can lead do renal dysfunction and the estimated lethal oral dose for humans is 350-3500 mg (WHO, 1996). Also, according to IARC carcinogen classification, cadmium and cadmium compounds were classified as Group 1, therefore carcinogenic to humans (IARC, 1993). In toxic amounts, this element generates Reactive Oxygen Species (ROS) and consequently alters intracellular machinery (Pourahmad & O'Brien, 2000). Also, Cd ions have strong affinity to sulfhydryl groups of several compounds, which could lead to undesirable bindings, for example, Cd complexes with metallothionein proteins (Kabata-Pendias, 2011).

The Decree-Law n. 103/2010 regulated the Environmental Quality Standard-Arithmetic Mean (EQS-AM) of Cd and their compounds in surface waters. The values are ≤0.08 and 0.25 $\mu g.L^{-1}$, depending on the water hardness for fresh water and for transitional, coastal and territorial waters the value is 0.2 $\mu g.L^{-1}$ (Decree-Law n. 103/2010). Also, cadmium and its compounds were reconsidered as priority hazardous substances (substance or toxic substance persistent, and susceptible to bioaccumulate at risk levels) by the European Community, within the Water Frame Work Directive (Directive 2013/39/EU).

Lead – Pb

Lead is a nonessential metal, also is the most common heavy metal found in the earth's crust, and it can be found in air, water, food (WHO, 1996) and mostly in soils due to human activities (Kabata-Pendias, 2011). The major use of lead is for lead-acid batteries. Also, it is used in solders, alloys, cables, chemicals, and for many other purposes. (Kabata-Pendias, 2011).

Lead is toxic, with accumulative behaviour, and regarding humans, fetus and children are the most susceptible to adverse health effects, such as on the central nervous system (WHO, 1996). Commission Directive 2003/40/EC of 16 May 2003, establishing the list, concentration limits and labelling requirements for the constituents of natural mineral waters also sets a maximum limit for lead in natural mineral water of 10 µg.L^{-1}. Also, lead and its compounds were reconsidered a priority pollutant by the European Community, within the Water Frame Work Directive (Directive 2013/39/EU). The Decree-Law n. 103/2010 regulated the Environmental Quality Standard-Arithmetic Mean (EQS-AM) of Pb and their compounds in surface waters. The value is 7.2 µg.L^{-1} for fresh, transitional, coastal and territorial waters.

1.3. Óbidos lagoon and metal contamination

Located in western Portugal, Óbidos Lagoon is a coastal lagoon near the city of Caldas da Rainha (**Figure 1.1**). This lagoon is connected to the sea by an artificial inlet and comprises 7 km^2 of wet area (Carvalho *et al*., 2006), and an average depth of 3 m, which makes them one of the biggest coastal lagoons in continental Portugal (Pedro *et al*., 2013)**.** This lagoon has a great economic interest due to human exploration, from fisheries and bivalve harvesting to recreational and human activities. Also, as a complex ecosystem, this lagoon as a great ecological importance. It serves as shelter for multiple aquatic birds species and is a nursery area for several species of fishes, namely with economic importance (Pedro *et al*., 2016).

Figure 1.1 – Location of the Óbidos Lagoon. The city of Caldas da Rainha on the right and the village of Óbidos on the bottom.

The Óbidos lagoon receives freshwater flows from different sources, namely the rivers *Cal*, *Arnóia*, *Real* and *Borraça*, as well as the *Ameal* ditch (Pedro *et al.*, 2016) and, for several years, this lagoon was the main receptor of urban discharges from several cities in the west region. In 2005, the urban loads of Óbidos, Carregal, Charneca, Caldas da Rainha and Foz do Arelho Waste Water Treatment Plant (WWTP), previously discharged to this lagoon, were deviated to the coastal adjacent waters via a submerged outfall. Nowadays, the only urban discharge to the Óbidos Lagoon is the Casalito WWTP (Malhadas *et al.*, 2014). However, the water flows from rivers are also a source of pollution. According to *Sistema Nacional de Informação de Recursos Hídricos* (SNIRH), the *Arnóia* river water is of mediocre quality and only suitable for irrigation, cooling and navigation purposes. Also, the continuous loads of Caldas da Rainha WWTP through *Cal* river, makes *Barrosa*'s Branch the most problematic area (Malhadas *et al.*, 2014). In brief, the continuous contamination and the deficient water renewal, typical of this environment, may slow down the dilution process and increase sediment accumulation, which leads to contaminants retention (Pedro *et al.*, 2013).

Several authors have been evaluating and monitoring the pollution and its impact in this lagoon. In 2014, Malhadas & Mateus applied a modeling system to relate analyzed nutrients in water before and after most urban discharges stopped. These authors observed that eutrophication levels were still elevated. Also, they suggested different hypothetical management scenarios and concluded that the main source of nutrient contamination were pig farms (Malhadas *et al.*, 2014). According to Pereira *et al*. (2014)

and Veiga *et al.* (2019), Óbidos Lagoon is also moderately contaminated by metals due to urban wastewaters, and agricultural and industrial runoffs from the surrounding region. Pereira *et al*. (2009b) evaluated metal contamination and eutrophication in Óbidos Lagoon on a seasonal basis and verified that *Barrosa*'s Branch (BB) is an impacted area due to discharges from *Cal* river. Pereira *et al*. (2014) used the algae *Ulva* spp. as a biomonitoring tool to study eutrophication and metal contamination (Mn, Cu, Fe, Pb and Cd) and verified that the algae tissues reflected the higher metal availability in BB station. Veiga *et al*. (2019) used the edible cockle *Cerastoderma edule* (Linnaeus, 1758) to evaluate contamination by Cd, Pb and Ni, and it was possible to detect the three metals at least once in the cockles, which means that these metals are available for human consumption. Carvalho *et al*., (2011) studied the main environmental drivers shaping temporal and spatial dynamics of macrobenthic communities at the lagoon. To better understand the complex link between abiotic and biotic interactions, Carvalho *et al*., (2011) used abundance and biomass data and verified how harsh the environment is in these biological patterns. Pereira *et al*., (2009a) studied the impact by metal contamination (Cu, Cd and Ni) and eutrophication in different areas of this lagoon. Also, those authors have reported biochemical effects in the crab *Carcinus maenas* (Linnaeus, 1758) as defense mechanisms to the environmental changes between BB and BSB stations.

1.4 *Hediste diversicolor* – characterization and bioindicator

The ragworm *Hediste diversicolor* (O.F. Müller, 1776) is a marine annelid, which belongs to the order Phyllodocida and to the family Nereididae (Scaps, 2002) (**Figure 1.2**). This species was previously named *Nereis diversicolor* due the similarities between the two genus. However, the genus *Nereis* lacks fused falcigers in the posterior neuropodia, which are present in the genus *Hediste* (Fong & Garthwaite, 1994). This species can be found in brackish or shallow waters in the North Temperate Zone, from both the European and The North American coast of the Atlantic Ocean. Its range extends from northern Europe and the Baltic Sea to Morocco and the Mediterranean, Caspian seas and by introduction in the Black sea (Smith,1997 *in* Scaps, 2002). In fact, as a result of its high ecological tolerance, this ragworm is found in all European estuaries, where it plays an important role in these ecosystems (Scaps, 2002) by contributing to the nutrients and contaminants biogeochemical cycles (Banta & Andersen, 2003; Buffet *et al.*, 2014).

This polychaete lives most of its life under the sediments and the behavior and characteristics of these animals make them suitable for pollutant analyses. The immature individuals of this species have a reddish brown colour. After growth and development, mature males assume a bright grass-green colour and females a darker green colour (Dales, 1950 *in* Scaps, 2002). In general, these organisms are available all year round and live in different subtracts, such as sandy muds, but also gravels and clays, where they build Y or U-shaped burrows (Esselink & Zwarts, 1989). *Hediste diversicolor* reproduces and feeds near the opening of its burrow. The adults are territorial, defending their territory against intruders. Burrows of different individuals are never connected, and territoriality is only exhibited inside the sediment (Lambert & Retière, 1987 *in* Scaps, 2002).

This species is gonochoristic and in rare cases, isolated females use viviparity and parthenogenic development of oocytes. The rupture of female dissepiment and disruption of male nephridies or dissepiment allows gametes liberation. The fertilized eggs remain in the maternal burrow and during incubation, larvae and post-larvae continue under the female's body at least for 10 days. Therefore, reproduction is followed by the organism's death, which makes them a monotelic species. In general, maturity takes between 1 and 2 years before spawning (Scaps, 2002). Spawning season could be variable and is controlled by temperature (5-11°C) and lunar periodicity. Variations already documented include a single spawning season, occurring in spring or summer, after a period of low temperatures in winter, but also, an extended breeding season with one or two peaks, or even spawning during the whole year (Scaps, 2002).

Luis & Passos (1995) quantified the diversity of lipids produced by *Hediste diversicolor* every month, during a whole year, and concluded that lipids were particularly important for reproduction in this species, especially triacylglycerol, which accumulates as an energetic reserve in oocytes. Also, energetic reserves are an indicator of contamination due its consumption under stresses (Durou *et al*., 2007; Pook *et al*., 2009). Therefore, analyses on lipid content might be a useful tool to better understand both the population and the environmental status.

Hediste diversicolor has different feeding tactics and, inevitably, variable preys and food items. This ragworm is known as a detritivore, also associated with bacteria ingestion, an herbivore (Costa *et al*. 2006) and a carnivore (Rönn *et al.*, 1988; Costa *et al*., 2006). For instance, Costa *et al*. (2006) analyzed the feeding patterns of *H. diversicolor* and found that they fed on different polychaete species, mostly from the family Nereididae. Also, multiple crustaceans, but mostly *Corophium* sp. were found as a source of food. In major quantities, Costa *et al.* (2006) found mucus, sand and vegetables detritus among the ingested materials. As predators, *H. diversicolor* can crawl on the sediment surface looking for food and catching it with their jaws. However, they are mostly detritivore and could use mucus secretions to collect food particles from the sediment (Scaps, 2002). The ragworm may also behave as a filter-feeder eating a secreted mucus filter that has captured phytoplankton and particles transported into the burrow by water circulation (Vedel, 1998). The filter-feeding activity is related and dependent on phytoplankton, decreasing when the phytoplankton concentrations are low (Vedel & Riisgård, 1993).The choice between these various feeding strategies is related to food availability, presence of predators, season and other local conditions such as tidal height (Esselink & Zwarts, 1989; Vedel *et al.*, 1994). By creating its galleries and by organic matter incorporation, *H. diversicolor* may increase the concentration of oxygen in the sediment (Kristensen *et al.*, 1992). Also, through their bioturbation activity these polychaetes play an important role in organic matter decomposition and contribute to the nutrients and contaminants biogeochemical cycles (Banta & Andersen, 2003; Buffet *et al.*, 2014).

The ragworm is capable of tolerating different environmental parameters. This animal survives in conditions of hypoxia (Scaps, 2002) and tolerates a different range of sediment's particle sizes, temperature and salinity, which allow them to settling in naturally-fluctuant environments, such as estuaries (Scaps, 2002; Bouraoui *et al*., 2016). The sediments of estuarine environments are rich in organic matter and pollutants (Ribeiro *et al*., 2018).Species like *Hediste diversicolor* feed from these sediments and predate other benthic organisms (Scaps, 2002; Costa *et al*., 2006). Also, as previously mentioned, this species may have a filter-feeder behavior and accumulate metals from water (Harley, 1950 *in* Scaps, 2002). As a result, this species apparently present detoxification mechanisms to protect them from metal toxicity (Bouraoui *et al*., 2016). Most invertebrates are included in low trophic levels which make them essential for metal transfer in food chains to higher trophic levels, including to economically important species (Buffet *et al*., 2014). *Hediste diversicolor* is an easy target for predators. In fact, birds have an important effect on *H. diversicolor* populations and shrimps, crabs and small fish are often implicated in the failure of larvae growth and survival (Scaps, 2002).

Due its characteristics, there are several studies using *H. diversicolor* as a bioindicator for pollution evaluation. According to McGeoch (1998) *in* Hodkinson & Jackson (2005), bioindicator is a species or group of species who reflects biotic and abiotic environment conditions. In other words, it is an organism used to evaluate synergetic and antagonistic impacts of different contaminants (Parmar *et al*., 2016). The good bioindicator species is capable of accumulating contaminants in concentrations higher than usual, which may be accompanied by alterations in the number of organisms in the population. They present characteristics of little ecological tolerance to contaminants and, at the same time, can present physiological, morphological or behavioral modifications due to the contaminants influence (Hodkinson & Jackson, 2005).

Berthet *et al.* (2003) evaluated metal contamination by Cd, Cu and Zn in *H. diversicolor* from several coastal sites in United Kingdom. Aydin-Onen *et al.* (2015) compared spatio-temporal distribution of metal contamination (Cd, Cr, Cu, Hg, Pb, and Zn) in the surface sediments and in tissues of *Hediste diversicolor* from Bafa Lake (Turkey). Durou *et al*., (2007) used physiological biomarkers (glycogen, lipid, protein), reproduction, growth and population structure of two distinct populations of the ragworm *H. diversicolor*, from two estuaries in France, with different levels of contamination. Those authors evaluated the health status of the populations in order to associate better health conditions to less contaminated estuaries and poorest health conditions to higher contaminated estuaries. Due to this, *H. diversicolor* has the necessary characteristics to be used as a bioindicator and consequently, in the last years, they have been intensely used as bioindicators, being useful to obtain important information about environmental changes.

1.5. Aims of the study

Óbidos lagoon has a great ecologic and economic potential, which leads to an extendedly anthropogenic exploitation, and inevitably, human activities are mostly the source of contamination by trace metals. These contaminants tend to accumulate and persist in the environment. Species in low trophic levels, such as *Hediste diversicolor* have the potential to bioaccumulate these trace metals. As they are prey for multiple species, such as fish, the contaminants may furtherly reach higher trophic levels and, eventually, may be consumed by humans. As a consequence, Óbidos lagoon is a vulnerable ecosystem where continued monitoring and a regular evaluation of the lagoon status are priorities.

In this context, and considering the bioindicator potential of this species, the present work aims to evaluate, on a seasonal basis, the pollution by trace metals (Cd, Cu, Fe, Mn, Pb and Zn) in the Óbidos Lagoon between autumn 2018 and spring 2019, using environmental samples and the polychaete *Hediste diversicolor* as a bioindicator. For this purpose, the next specific objectives were:

- Quantification of trace metals in environmental samples of water and sediments, collected at the Óbidos Lagoon in locations with a different degree of contamination.

- Quantification of trace metals in biological samples of the bioindicator species *Hediste diversicolor* in locations with different contamination levels.

- Evaluation of trace metals accumulation in biological samples of *Hediste diversicolor* using bioaccumulation and concentration factors.

- Analyses of the effects of trace metals on the physiological and biochemical (lipids content) parameters, on the biometry and on the bioaccumulation ability.

2. Materials and Methods

2.1. Study Area

The Óbidos Lagoon is a coastal lagoon located in western Portugal (39° 24′ N, 9° 17′W) near the city of *Caldas da Rainha* and *Óbidos*. With about 7 km^2 of wet area, the average depth is of 2-3 m and this lagoon is one of the biggest coastal lagoons in continental Portugal (Pedro *et al.*, 2013). This lagoon is connected to the sea by an artificial inlet which has suffered several alterations in the last decades and is under anthropogenic influence (Ferreira *et al.*, 2009). During 2018 the lagoon lost the contact with the sea two times, in May and in the end of December. However, in both times the inlet was reopened in a few days.

Freshwater inputs come from the rivers *Cal*, *Arnóia* and *Real*, *Borraça* and from the *Ameal* ditch, taking agricultural runoffs through the rivers, mainly in winter (Freire *et al.*, 2018; Veiga *et al.*, 2019). The lagoon is distinguished by two main regions with different geological and hydrological characteristics, the lower and the upper lagoon (Malhadas *et al.*, 2014; Pedro *et al.*, 2016). The lower lagoon is connected to the ocean and is characterized by sand banks and narrow channels (Pedro *et al.*, 2016), which have naturally changed, but also suffered human interventions. This area is characterized by coarser sediments with low affinity for metals and by a better water quality as well (Pereira *et al.*, 2010). The upper lagoon, where sediments are muddier and water has higher residence times, which reduces dilution processes and enhances sediment retention of contaminants (Pereira *et al.*, 2010; Pedro *et al.*, 2016), is characterized by a large shallow basin, with two branches, *Barrosa* and *Bom Sucesso*, (BB and BSB respectively) and a small embayment, *Poça das Ferrarias* (PF) (Malhadas *et al.*, 2014). In the present study, four sampling stations located in the upper lagoon, were selected as study sites (**Figure 2.1**).

Figure 2.1 – Location of the study sampling sites in the Óbidos Lagoon: BB – *Barrosa*'s Branch; AR – *Arnóia* and *Real* rivers; CM – *Covão dos Musaranhos*; and PF – *Poça das Ferrarias*.

The first sampling station was located in *Barrosa*'s Branch (BB) (39°23'58.9"N; 9°11'14.7"W). This is the shallowest arm of the lagoon with a mean depth of 0.5 – 1 m. The water circulation is mostly driven by the tides and by a small affluent, the *Cal* river (0.14 $m^3.s^{-1}$ average flow) (Malhadas *et al.*, 2014). According to the Portuguese classification of freshwater systems (IST/IPIMAR, 2008), the water of this river has reduced quality. It is also recorded that, in the last decades, this river receives domestic effluents from the rivers in *Caldas da Rainha* and *Gaeiras*. This lagoon area presents high levels of nutrients and has been classified as eutrophic, presenting high metal levels in the water column as well (Pereira *et al*., 2010).

The following sampling station (39°23'59.9"N; 9°12'18.8"W) was located between two branches where *Arnóia* and *Real* (3 $m^3.s^{-1}$ average flow) rivers drain to the lagoon (Malhadas *et al*. 2014), hereafter called as station AR. According to Malhadas *et al.* (2014), these rivers are most probably contaminated by agricultural pollution and are known to contribute with about 90% of the freshwater discharges into the lagoon (Malhadas *et al.*, 2009).

The next sampling station was located at the *Bom Sucesso* branch in a site known as *Covão dos Musaranhos* (CM) (39°23’16.7”N; 9°13’32.8”W). This branch receives a lower freshwater flow than BB station, from the Ameal ditch (0.08 $m^3.s^{-1}$ average flow) (Malhadas *et al.*, 2014). This area is characterized by a better water quality and by sandy bottom sediments (Pereira *et al.*, 2009b) with a low affinity for metals (Carvalho *et al.*, 2006). According to the Portuguese classification of freshwater systems it has a better water quality (SNIRH – *Sistema Nacional de Informação de Recursos Hídricos*).

The last station was *Poça das Ferrarias* (39°24’03.6”N; 9°13’13.0”W), hereafter called as station PF. It is a small bay with a narrow opening. Therefore, water flows are reduced, resulting in confined waters. The tides’ influence in this site is dependent of multiple factors. There is also a wooden bridge, which was fixed and modified by the end of March of 2019.

2.2. Sampling program

Environmental (water and sediments) and biological (*H. diversicolor*) samples were collected during low tide, in the periods of spring tides, in the intertidal areas of four different stations (BB, AR, CM and PF) and in four moments, representing each one of the seasons (Summer, Autumn, Winter and Spring). In each sampling station, and at each season under study, the samples were collected following the same procedures.

For water samples, 1.5 L of surface water was collected in a plastic bottle (PET-Polyethylene Terephthalate) from 3 different places at each sampling station, for metal quantification. The temperature, pH, and salinity of the water in each station and season, were measured with a portable multiparameter probe HANNA Hi 9828 (Woonsockets, EUA). Both procedures occurred at approximately 50 cm deep. For sediment analyses three different replicas from the same area were collected by hand, at 5 cm deep Samples were maintained in plastic bags until laboratory analyses. For biological sampling were collected 35 individuals of *Hediste diversicolor* in each station and moment, and the specimens were maintained in plastic flasks in a portable icebox, until laboratory analyses. For the further analyses, 30 individuals were used for biometry, 10 of them for metal analyses, and 7 of these 10 specimens were also used for lipids analyses. To notice, lipids analyses were only performed to individuals from AR and CM stations. CM station is known to be less contaminated and AR station is known to be probably contaminated by agricultural pollution (Malhadas *et al.*, 2014).

The mensal precipitation data during the sampling months was obtained from SNIRH.

2.3. Samples treatment

2.3.1 Water

The samples of water were filtered in vacuum with an acetate membrane Whatman OE 67 (pore 45 µm and 45 mm diameter, Germany). To perform the analyses in AAS, the pH of the filtered water was verified, and the samples were acidified, until pH<2, with nitric acid (HNO_3 69.6%, AnalaR NORMAPUR, VWR Pro Lab Chemicals, France). Finally, a volume of 0.05 L of water from each of the 3 replicated samples was stored in plastic flasks at -18°C for future analyses of the presence of dissolved metals. The resultant filtration membranes Whatman OE 67 (pore 45 µm and 45 mm diameter, Germany) were also frozen for future digestion and for future quantification of the trace metals present in the suspended matter (Pedro *et al*., 2016).

2.3.2 Sediment

The sediment samples, collected as previously explained, were used for the quantification of trace metals and for the determination of the organic matter in the sediments. For the quantification of the organic matter content, the samples were divided in weight crucibles and were dried in a drying oven (Memmert oven, Schwabach, Germany) at 60ºC, for at least 48h. After that, crucibles were weighted (AE ADAM, PGL 3002, Milton Keynes, England) and left in a desiccator to avoid humidity. Afterwards the crucibles were placed in a muffle (Nabertherm GmbH, Germany) for 8h at 450ºC and maintained in a desiccator. The weight of the sediments was determined with the formula below, where organic matter content (OMC), in percentage (%), was quantified using the sediment weights before and after the combustion in the muffle.

$$OMC\ \% = \frac{Dry\ weight - Ash\ weight}{Dry\ weight} \times 100$$

For a better characterization of the environmental samples, a granulometry analysis was performed to distinguish sediments from each station. As mentioned before, all sediment samples were dried 8h in a muffle at 450ºC and after that, granulometry was analyzed in a particle size analysis equipment. The laser granulometer (Beckman-Coulter LS230, USA) was used for sediments below 63 µm and for sediments above 63 µm the analysis was performed in an analytical sieve shaker (Retsch GmbH, AS200 control G, Germany).

2.3.3 *Hediste diversicolor*

The specimens of *H. diversicolor* were found buried in the sediments, where they construct their galleries. As a result, they were gently digged by hand to avoid the animals' fragmentation. The specimens were stored in plastic flasks with algae, collected from their habitat, in a portable icebox and transported to the laboratory (Mouneyrac *et al.*, 2010).

At each station 35 specimens were randomly collected by hand and, at the laboratory, they were separated in four different aquariums, with brackish water (salinity 25), constant aeration and marble balls. They were maintained about 72 hours in the dark for depuration, similarly as in Kalman *et al.* (2010). After that, the animals were weighted (wet weight) (Sartorius, ENTRIS 224I-1S), their body size (in cm) was measured and furtherly the animals were separated in flasks and stored at -4 °C until biometric analyses (see section 2.5 for further details). All specimens were afterwards stored at -80 °C (Thermo Scientific -86°C freezer, HERAFREEZE HFU T series, USA), until lyophilization. Finally, the ragworms were lyophilized (SCANVAC cool safe 55-4, Denmark) and 10 individuals were homogenized for metal analyzes and for the quantification of lipids content.

Biometrical characterization and sex determination

Hediste diversicolor specimens have a soft body and can easily lose body parts by accident, which makes the calculation of health and condition index of these populations more difficult. Durou *et al*. (2008) and Mouneyrac *et al*. (2010) have suggested the use of size–weight relationships as tools to evaluate the population status. In this context, the measurement of the first three segments (peristomium, prostomium and first chaetiger), named L3, as well as the length of the first chaetiger and the body size are recommended. In the present study, all the animals collected at each sampling moment and in each sampling station (N=35), were subjected individually to the following measurements: each specimen was carefully stretched and the full length was measured from anus to the head with a scale ruler, in cm. Also, the wet weight (in g) was determined (Sartorius, ENTRIS 224I-1S, Germany) and registered. Each individual was analyzed with a trinocular stereo microscope (Stemi 2000-C, Carl Zeiss Jena GmbH, Germany) and the software ZEN lite (ZEN 2011 - Blue edition, Carl Zeiss) and the measurements of the prostomium, peristomium and 1st chaetiger (L3) length were performed in mm. Also, the first segment, or 1st chaetiger width was determined in mm.

Using the binocular microscope (Zeiss Stemi 305, USA), the sex of each animal was determined. A portion of coelomic fluid was extracted from the organisms, with a needle close to the 30th segment for oocytes liberation and observation, similarly as in Durou & Mouneyrac (2007). The individuals are sensitive and were frozen, so it was not possible to correctly identify spermatogonia plates in males, which were damaged. Therefore, when there was no evidence of oocytes the specimen was classified as indeterminate, because it was not possible to distinguish the males with certainty, so indeterminate specimens could be male or female.

2.3.4 Samples acid digestion

To obtain the total metal content in the suspended matter, sediments and in *Hediste diversicolor'*s samples, acid digestions were performed with a high-performance microwave digestion system (Milestone connect, MA182-001 Ethos up, Italy). The digestions were performed according to the protocols established by the equipment fabricant. The filtration membranes Whatman OE 67 (pore 45 µm and 45 mm diameter, Germany), for the suspended metals quantification, and portions of 10 different individuals of *Hediste diversicolor,* from each station and each sampling moment, were digested according to the protocol EPA 3051 in nitric acid 65% (HNO_3 69.6%, AnalaR NORMAPUR, VWR pro lab chemicals, France) during 1h, until 180 °C. After digestion, the samples were filtered with paper filters Whatman 41 (55 mm diameter, Germany), to avoid remained silicate particles, and furtherly, were diluted to a final volume of 50 mL, with distilled water type 2. The pH was measured with a pH meter (WTW inoLab ®, pH 7110, Germany) and all digested samples were maintained at pH<2, stored in plastic flasks and frozen at -18 °C. Sediment digestion was performed with hydrogen peroxide 30% (H_2O_2 30%, EMSURE® ISO, Merck KGaA, Germany) and nitric acid 65% in a 1:9 dilution. Between each digestion a cleaning protocol was performed for equipment decontamination during 1 hour in nitric acid diluted to 32.5%.

2.4 Metals quantification

All the materials used in this methodology had to be made from plastic and free of metal contaminants. Therefore, they were first washed with 3% Derquim LM 02 (Panreac Química SAU, Spain) for 24h, maintained in distilled water for 24h and furtherly left in HNO_3 25% for 24h. After the acidic wash, the material was left for 24h in ultrapure water, and then it was dried in a drying oven at 60^{o}C (Memmert oven, Schwabach, Germany).

Metal analyses were performed by Atomic Absorption Spectrometry (AAS) (Thermo Scientific ICE 3500, Thermo unicam, Portugal). This method follows the principle of Lambert-Beer law, which means that absorption is proportional to the concentration of the metal present. Like such, standard metal solutions, daily prepared using metal stock solutions and distilled water type 2 (Elix® Advantage pure water, type 2), were used for each metal to obtain the respective calibration curves. The metal stock solutions used were the following: Cadmium standard solution (1000±0.0002 g.L^{-1} AA Panreac Quimica SLU, EU); Copper standard solution traceable to SRM from NIST $Cu(NO_3)_2$ in HNO_3 0.5 mol.L^{-1} 1000 mg.L^{-1} Cu (Certipur®, Merck KGaA, Germany); Iron standard solution traceable to SRM from NIST $Fe(NO_3)_2$ in HNO_3 0.5 mol.L^{-1} 1000 mg.L^{-1} Fe (Certipur®, Merck KGaA, Germany); Lead AAS Standard 1000 mg.L^{-1} in nitric acid 2% (AVS TITRINORM, VWR international, Belgium); Manganese standard solution traceable to SRM from NIST $Mn(NO_3)_2$ in HNO_3 0.5 mol.L^{-1} 1000 mg.L^{-1} Mn (Certipur®, Merck KGaA, Germany); and Zinc standard solution traceable to SRM from NIST $Zn(NO_3)_2$ in HNO_3 5 mol.L^{-1} 1000 mg.L^{-1} Zn (Certipur®, Merck KGaA, Germany).

To determinate trace metal concentrations it was used the standard addition method, with correlations between 0.9913 and 0.9999. For all samples was used a sample blank of 1% of HNO_3 (HNO_3 69.6%, AnalaR NORMAPUR, VWR pro lab chemicals, France) in ultrapure water. For sediment samples was used also a blank with 1% of HNO_3 (HNO_3 69.6%, AnalaR NORMAPUR, VWR pro lab chemicals, France) in distillate water type 2 and hydrogen peroxide 30% (H_2O_2 30%, EMSURE® ISO, Merck KGaA, Germany).

For trace metals found in lower amounts, quantification had to be performed by Atomic Absorption Spectrometry with Graphite Furnace Atomization (GFAAS). For all environment and biological samples, the elements cadmium and lead were analysed by GFAAS and their concentrations are expressed in µg.L^{-1}. The detection limits for the tested elements in GFAAS are: cadmium, 0.001 µg.L^{-1} and Pb 0.06 µg.L^{-1}. Trace metals found in higher amounts were quantified by Flame Atomic Absorption Spectrometry (FAAS). The elements copper, iron, manganese and zinc were analyzed by FAAS for all environmental and biological samples, and their concentrations are expressed in mg.L^{-1}. The detection limits for the tested elements in FAAS are 0.4 µg.L^{-1} for Cu; 0.1 µg.L^{-1} for Fe; 0.035 µg.L^{-1} for Mn; and 0.1 µg.L^{-1} for Zn. The biological samples consisted of 10 animals per sampling station, which were collected at each sampling moment. Environmental samples consisted of three replicas for each station and season.

To perform the analyses in AAS, all the samples were acidified with HNO_3 until a pH<2 was reached. Also, the matrix modifier Magnesium nitrate (Matrix Modifier Solution, 1% $Mg(NO_3)_2$ in 2% HNO_3 Specture®, ThermoFisher (KAndel) GmbH, Germany) was added to all samples for all elements. The matrix modifier forms a stable compound with the element of interest, that can be ashed in higher temperatures allowing the use of highly ashing temperatures to avoid volatile compounds loss.

2.5 Lipid Analysis

To analyze the lipid content of the animals, 7 individuals from the AR and CM stations were selected from all seasons. Lipid extraction was performed with chloroform/methanol ($CHCl_3$ stabilized with 0,6% ethanol, AnalaR NORMAPUR, VWR® BDH® Chemicals, France; CH_3OH, HiPerSolv. CHROMANORN, Prolabo ® VWR, France) and sulphuric acid (H_2SO_4 95% VWR Prolabo® Chemicals, France) at 200°C in a drying oven (Memmert GmbH, Germany) according to the Bligh and Dyer method (Bligh & Dyer, 1959). The quantification of lipid content was carried by measuring absorbance at 340 nm with a standard curve of triglyceride tripalmitin (Glycerol Tripalmitate ($C_{51}H_{98}O_6$ 98%, Alfa Aesar GmbH & Co, Karlsruhe, Germany)) in a microplate reader (Microplate reader Epoch 2C Biotek Instruments, Inc, USA) using the software Gen5 3.02, similarly as in Pook *et al.*, (2009) for this species.

2.6 Data analysis

2.6.1 Metals bioaccumulation abilities

As an important tool for pollution evaluation, the ability of contaminants accumulation by the organisms can be assessed with different bioaccumulation models, using different chemical and biological parameters. The basic equation of bioaccumulation establishes a mass balance of contaminants in the organisms and in the environment (Amiard-Triquet *et al.*, 2015).

Hediste diversicolor lives and feeds under and near the sediment surface. The Bioaccumulation Factor (BAF) creates a relation between the accumulated contaminants in the organism and in the sediment. For each station and sampling moment, the Bioaccumulation Factor (BAF) of each of the *H. diversicolor*'s specimens was evaluated. This is the ratio of a contaminant concentration in the organism to its contamination in the environment and is calculated using the following equation:

$$\frac{C_o}{C_s}$$

Where,C_o – metal concentration in the organism in µg.g^{-1} or mg.kg^{-1} and C_s – metal concentration in the sediment in µg.g^{-1} or mg.kg^{-1}.

As mentioned before, sometimes this species acts also as a filter-feeder and then could accumulate metals directly from the water. In this context, the use of the Concentration Factor (CF) is also a way to evaluate the contaminants accumulation in *H. diversicolor* (Bryan & Hummerstone, 1973; Alquezar *et al.*, 2007). The Concentration Factor (CF) is the ratio of a contaminant concentration in the organism to its contamination in the water. This factor was calculated for each animal from each sampling moment, by applying the following equation:

$$\frac{C_o}{C_w}$$

Where, C_o – metal concentration in the organism in µg.g^{-1} or mg.kg^{-1} and C_w – concentration of the same metal in the seawater in µg.L^{-1} or mg.L^{-1}.

2.6.2 Statistical analyses

The statistical analyses were all performed in the statistical software (IBM® SPSS® Statistics 25). Previous to any statistical analysis, all the data was tested for normality (applying the non-parametric test Kolmogorov-Smirnov) and homogeneity of variance (using the Levene test) and transformed, when necessary. However, if the transformed data maintained heterogeneity, analyses were performed on the untransformed data, since analysis of variance is quite robust to departures from their assumptions (Underwood, 1997).

Two-way ANOVAS or the Mann-Whitney test were used to test sampling sites and seasonal effects in (i) the physicochemical variables water temperature, pH, OMC, precipitation and salinity; (ii) the metal concentration in the environment and biological samples and (iii) *in Hediste diversicolor* biologic responses (Biometric, lipids content, BAF and CF). Post-hoc tests were used to analyse interactions between factors. When interaction was observed, the Bonferroni test was used and in the absence of interaction was used the Tukey HSD test.

Spearman test was performed to verify the presence of correlations between (i) bioaccumulated metals in *H. diversicolor* and the metals found in the environmental samples (water and sediment); (ii) bioaccumulated metals and the biometric variables and lipid content. The strength of the correlations was evaluated according to the limits established in Fowler et al., (1998) where ρ-spearman values between 0.40 and 0.69 correspond to modest correlations; ρ-spearman values between 0.70 and 0.89 correspond to strong correlations; and ρ-spearman values between 0.90 and 1.00 correspond to very strong correlations. To notice, only the correlations with ρ-values < 0.05 and ρ-spearman > 0.5 were considered.

3. Results

3.1 Sampling sites – Physical-chemical characterization

At all sampling stations the water temperature was the lowest in winter as compared to the other seasons (**Figure 3.1A**) and significant statistical differences were found to corroborate this difference (Mann-Whitney test: U – 0.000; ρ-value – 0.021). Also, water temperatures were relatively higher in summer, when compared with autumn. The statistical test also showed significant differences between summer and autumn (Mann-Whitney test: U – 0.000; ρ-value – 0.021).

Comparing with the other stations, the sampling site *Poça das Ferrarias* (PF) presented the highest water temperatures in summer and winter, 30.1 °C and 15.0 °C, respectively, while the *Barrosa's* Branch (BB) presented the lowest water temperatures during those seasons, 21.0°C and 12.0°C respectively (**Figure 3.1A**). Also, BB station presented the highest temperatures during autumn and spring, 20.6 °C and 22.6 °C, respectively (**Figure 3.1A**), although statistical differences between sampling stations were not observed.

In winter the salinity at *Barrosa's* Branch (BB) and *Arnóia* and *Real* rivers (AR) presented the lowest values compared with the other seasons and stations (15 and 5.33, respectively). In contrast, at *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) the salinity presented much higher values and was never bellow 27 (**Figure 3.1B**). The statistical test confirms the statistically significant differences between the AR and CM stations (Mann-Whitney test: U – 0.000; ρ-value – 0.021) and between AR and PF stations (Mann-Whitney test: U – 1.000; ρ-value – 0.043).

The *Barrosa's* Branch (BB) shows the highest pH range between seasons, with values ranging from 5.32 in summer and 8.87 in spring. In all the sample sites, the pH during summer is clearly lower than the pH in the other seasons, and highest in spring (**Figure 3.1B**). However, the statistical test only shows significant differences between the spring and the summer and between spring and autumn (Mann-Whitney test: U – 0.000; ρ-values – 0.021).

As expected, precipitation (mm) was highest in autumn (46.6 mm ± 26.7), and winter (30.9 mm ± 8.1), followed by spring (12.3 mm ± 4.2), and almost absent in the summer (10.0 mm ± 3.1) (**Figure 3.1C**).

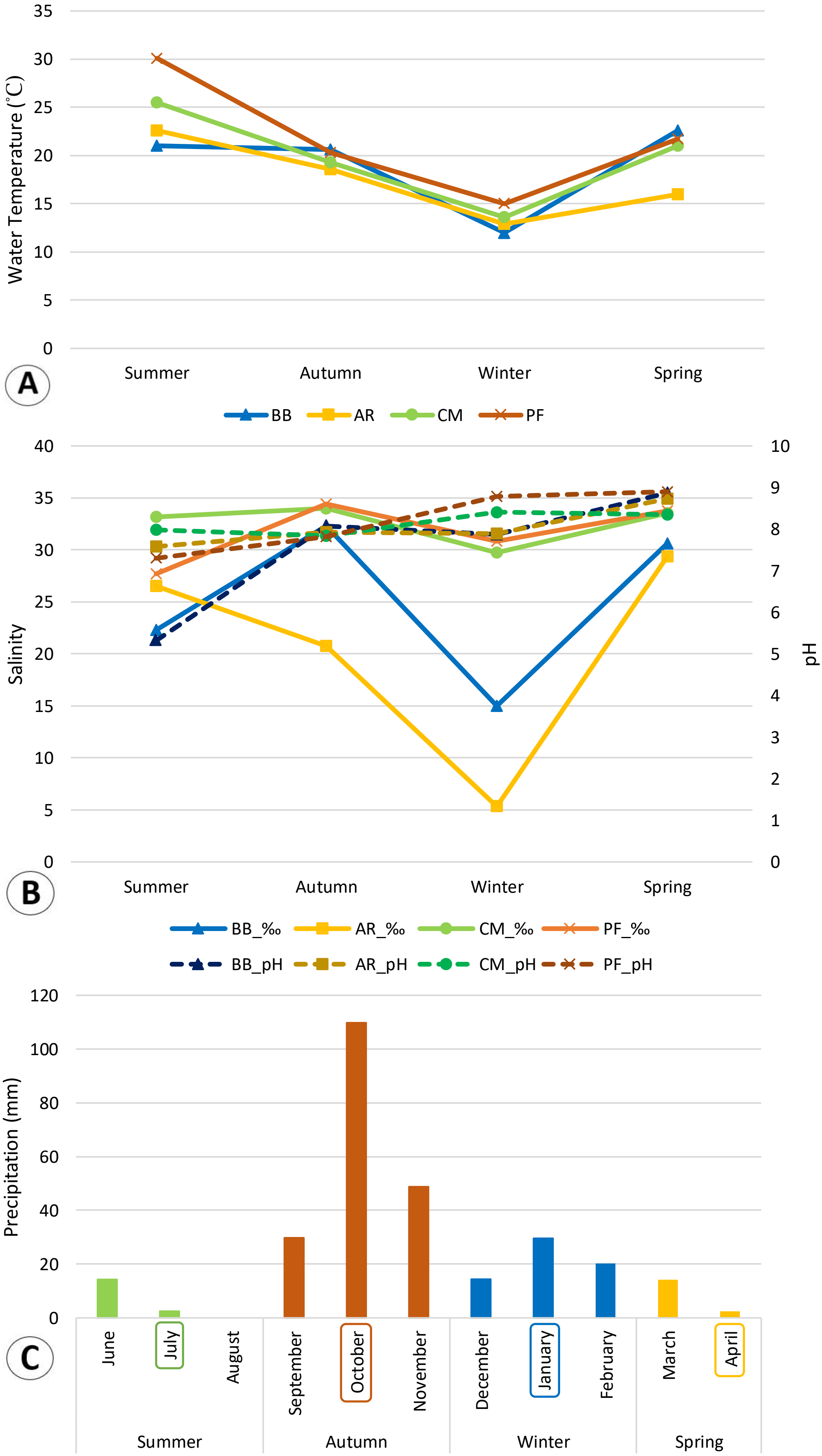

Figure 3.1 – Physicochemical parameters in the sampling sites at Óbidos Lagoon during the study period. A – Water Temperature (°C); B – Salinity and pH; and C – precipitation (mm). Sampling sites: BB – *Barrosa*'s Branch; AR – *Arnóia* and *Real* rivers; CM – *Covão dos Musaranhos*; PF – *Poça das Ferrarias*. The squared months correspond to the period of sampling: 26th of july, 23th of october, 21th of January and 16th of April.

The granulometry of the sediment is important for a better characterization of the sampling sites. The dominant particles at BB and PF stations were mainly silts and at AR and CM station the major particles were sand. In both cases, silts and sand represented over than 60% of total particles (**Figure 3.2**).

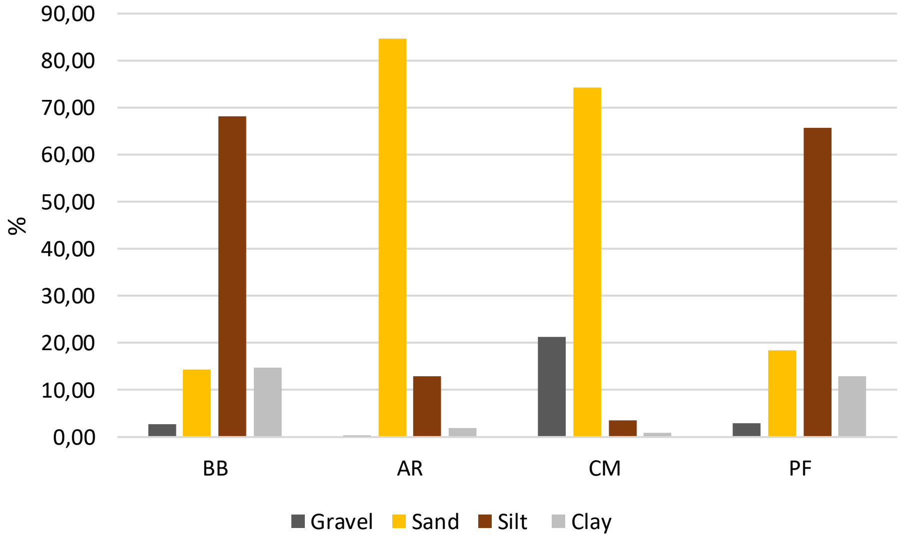

Figure 3.2 – Granulometry characterization (gravel, sand, silt and clay in percent %) of the sediment collected at Óbidos Lagoon. Sampling sites: *Barrosa'*s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF).

The organic matter content (OMC) from the sediment samples in *Barrosa'*s Branch (BB), *Arnóia* and *Real* rivers (AR) and *Covão dos Musaranhos* (CM) showed only small differences between seasons. On the other hand, at *Poça das Ferrarias* (PF) the highest level of organic matter content was observed in autumn (5.69 % ± 1.27) with a clear discrepancy compared with summer (0.92 % ± 0.22) and spring (1.10 % ± 0.50) (**Figure 3.3**). The Two-Way ANOVA test revealed effects between seasons and stations in the organic matter content (ρ-value – 0.000, **Attachment 1**). Also, the post-hoc test corroborates these OMC results, since statistically significant differences were observed at PF between autumn and the other three seasons (Bonferroni test: ρ-values – 0.000, **Attachment 1**). In the summer the OMC of the sediments was higher at BB as compared to the other stations, which was also confirmed by the post-hoc test (Bonferroni test: ρ-values between 0.000 and 0.001, **Attachment 1**). Also, in the spring the OMC at BB station presented higher values compared to the other stations, as confirmed by the post-hoc test (Bonferroni test: ρ-values between 0.005 and 0.018). In the winter, the OMC at AR station was significantly different from the OMC of the sediments at BB and PF stations (Bonferroni test: ρ-values between 0.003 and 0.005, respectively, **Attachment 1**).

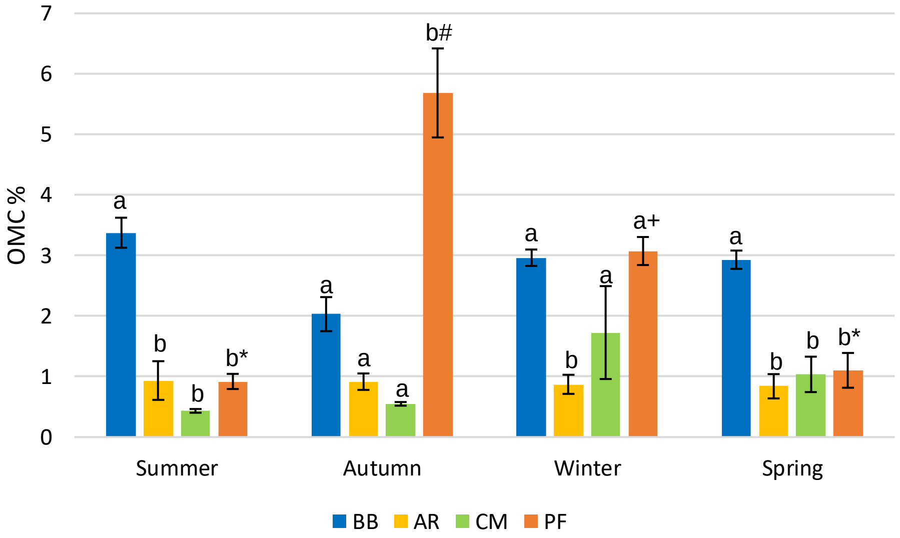

Figure 3.3 – Organic matter content (OMC) of the sediments, in percentage, at Óbidos Lagoon (N=3 for each station and season), during the study period. All values were expressed as mean and standard error expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR*), Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (*, # and +) indicate significant differences between seasons in the different stations and the letters (a and b) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni test); absence indicates that no significant differences were observed.

3.2 Metals quantification

3.2.1 Environmental samples – Sediment and water

Copper – Cu

The metal copper (Cu) was not detected dissolved in water samples, while suspended copper was found in all seasons although at different sampling stations. Also, copper was found in the sediments in all seasons and all stations, except once at CM station.

In autumn, suspended Cu was detected at all stations except for PF, while on the contrary in spring suspended Cu was only detected at PF station. The highest concentrations of suspended Cu were found in winter at BB station (0.1119 mg.L^{-1} ± 0.0552) and in autumn, at BB (0.0876 mg.L^{-1} ± 0.0000) and AR (0.1071 mg.L^{-1} ± 0.0040) stations (**Figure 3.4A**). The Two-way ANOVA procedure revealed an effect between seasons and stations in the presence of suspended Cu in water samples (ρ-value –

0.016, **Attachment 3**). The post-hoc test showed significant differences between summer and winter, and winter and spring at BB station (Bonferroni test: ρ-values – 0.021, **Attachment 3**). At AR station an effect of seasons was also observed, with differences between autumn and winter and autumn and spring (Bonferroni test: ρ-values – 0.029, **Attachment 3**). The lowest concentrations detected were found at PF station in spring (0.0133 mg.L^{-1} ± 0.0073), followed by AR, CM and PF in summer, autumn and winter, respectively (0.0341 mg.L^{-1} ± 0.0278) (**Figure 3.4A**). The post-hoc test showed significant differences in the autumn between AR and PF stations (Bonferroni test: ρ-value – 0.029, **Attachment 3**) and in the winter between BB and AR stations and BB and CM stations (Bonferroni test: ρ-values – 0.021, **Attachment 3**). Also, the following pattern was observed in each one of the sampling stations: concentrations of Cu suspended in the water samples above the detection limit of the analytical equipment used were only observed in two consecutive seasons at each sampling station, being not detectable afterwards, at AR and CM stations suspended Cu was only found in the summer and autumn, while at BB station it was found only in autumn and winter and at PF station it was only found in winter and spring (**Figure 3.4A**).

Copper was present in the sediments at all seasons and at all sampling stations, except in summer at CM (**Figure 3.4B**). The concentrations of this metal at BB station were the highest in all seasons as compared to the other stations, followed by PF station. Actually, Two-Way ANOVA test results showed effects between seasons and stations in copper concentrations in the sediment (ρ-value – 0.008, **Attachment 4**). Also, the post-hoc test for seasons showed statistically significant differences between BB and the other three stations in all seasons (Bonferroni test: ρ-values – 0.000, **Attachment 4**) except at PF station in the winter (Bonferroni test: ρ-value – 0.048, **Attachment 4**) and in the autumn no significant differences were found between these two stations. The highest concentrations in sediment at BB station were found in summer (55.37 mg.kg^{-1} ± 7.77) and the lowest in the autumn (31.41 mg.kg^{-1} ± 3.12) (**Figure 3.4B**). In fact, the post-hoc test showed that at BB summer and autumn were statistically different (Bonferroni test: ρ-value – 0.005, **Attachment 4**). At PF the lowest concentration of Cu in sediments was observed in summer (2.06 mg.kg^{-1} ± 0.87) and the highest in the winter (21.69 mg.kg^{-1} ± 8.38) (**Figure 3.4B**), which were confirmed to be significantly different (Bonferroni test: ρ-value – 0.029; **Attachment 4**).

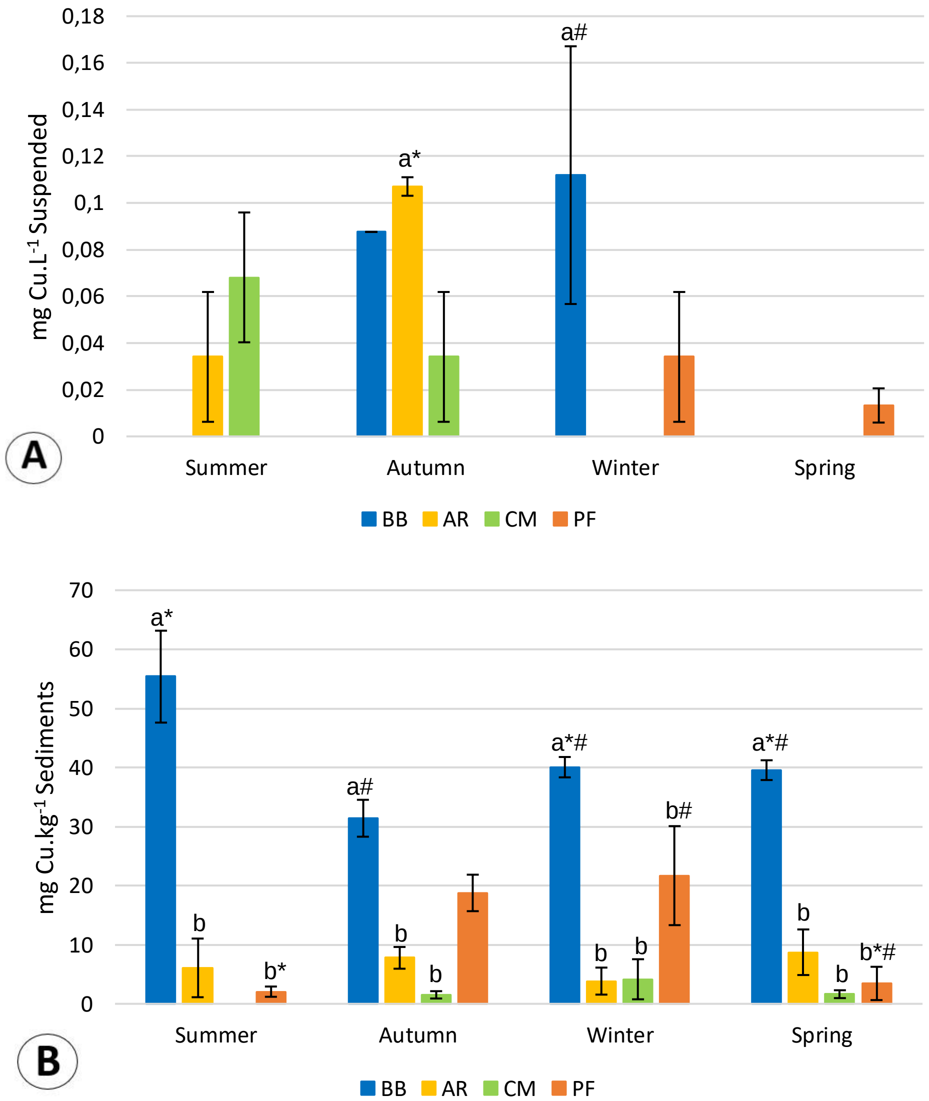

Figure 3.4 – Concentrations of Copper (Cu) in the environmental samples at the Óbidos Lagoon during the study period. (A) Total Cu suspended in the water column, in miligrams per litre (N=3, except for autumn at BB station); and (B) Total Cu in sediments, in miligrams per kilogram (N=3 in each season and station). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (* and #) indicate significant differences between seasons in the different stations and the letters (a and b) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni test); absence indicates that no significant differences were observed.

Iron – Fe

The element iron (Fe) was detected in all environmental samples from all stations and seasons.

The dissolved Fe was detected in water samples in all sampling sites and seasons, except for PF station in the autumn. The highest concentrations were found in winter at BB (0.0579 mg.L^{-1} ± 0.0000) and AR (0.0502 mg.L^{-1} ± 0.0063) stations. At PF station higher dissolved concentrations were found in the winter and spring (0.0347 mg.L^{-1} ± 0.0000, **Figure 3.5A**) followed by summer (0.0116 mg.L^{-1} ± 0.0095, **Figure 3.5A**) and the Two-Way ANOVA showed effects between stations and seasons in the dissolved iron (ρ-value – 0.038, **Attachment 2**). In addition, the post-hoc test revealed significant differences at BB station between winter and spring (Bonferroni test: ρ-value – 0.003, **Attachment 2**) and at AR station between winter and summer (Bonferroni test: ρ-value – 0.003, **Attachment 2**). As mentioned before, at PF station higher dissolved concentrations were found in the winter and spring (**Figure 3.5A**) and the post-hoc test supported this showing significant differences at PF station between the autumn and the winter and spring (Bonferroni test: ρ-values – 0.008, **Attachment 2**). The statistical test showed interactions between seasons and stations, however only significant differences were found between AR and PF stations in the autumn (Bonferroni test: ρ-value – 0.008, **Attachment 2**).

The highest concentrations of suspended Fe in the water column were found at BB and AR stations in autumn and winter. Also, the lowest values were found in summer and spring. On the contrary, at PF station the highest value was found in the spring. The concentrations of suspended Fe at CM station were lowest in winter and spring comparing with summer and autumn (**Figure 3.5B**). However, none of these values showed statistically significant differences between the studied factors.

For Fe concentration in sediment samples the Two-Way ANOVA only revealed effects between stations (ρ-value – 0.000). In fact, the concentrations found at BB station were significantly higher than at the other three stations (Tukey HSD test: ρ-values – 0.000, **Attachment 4**), in all seasons, and the highest value was found in summer (58067 mg.kg^{-1} ± 1.32) (**Figure 3.5C**). CM station showed the lowest values in all seasons, comparing with the other stations. The station PF presented the highest values in autumn and winter, while AR station showed similar values in the four seasons under study.

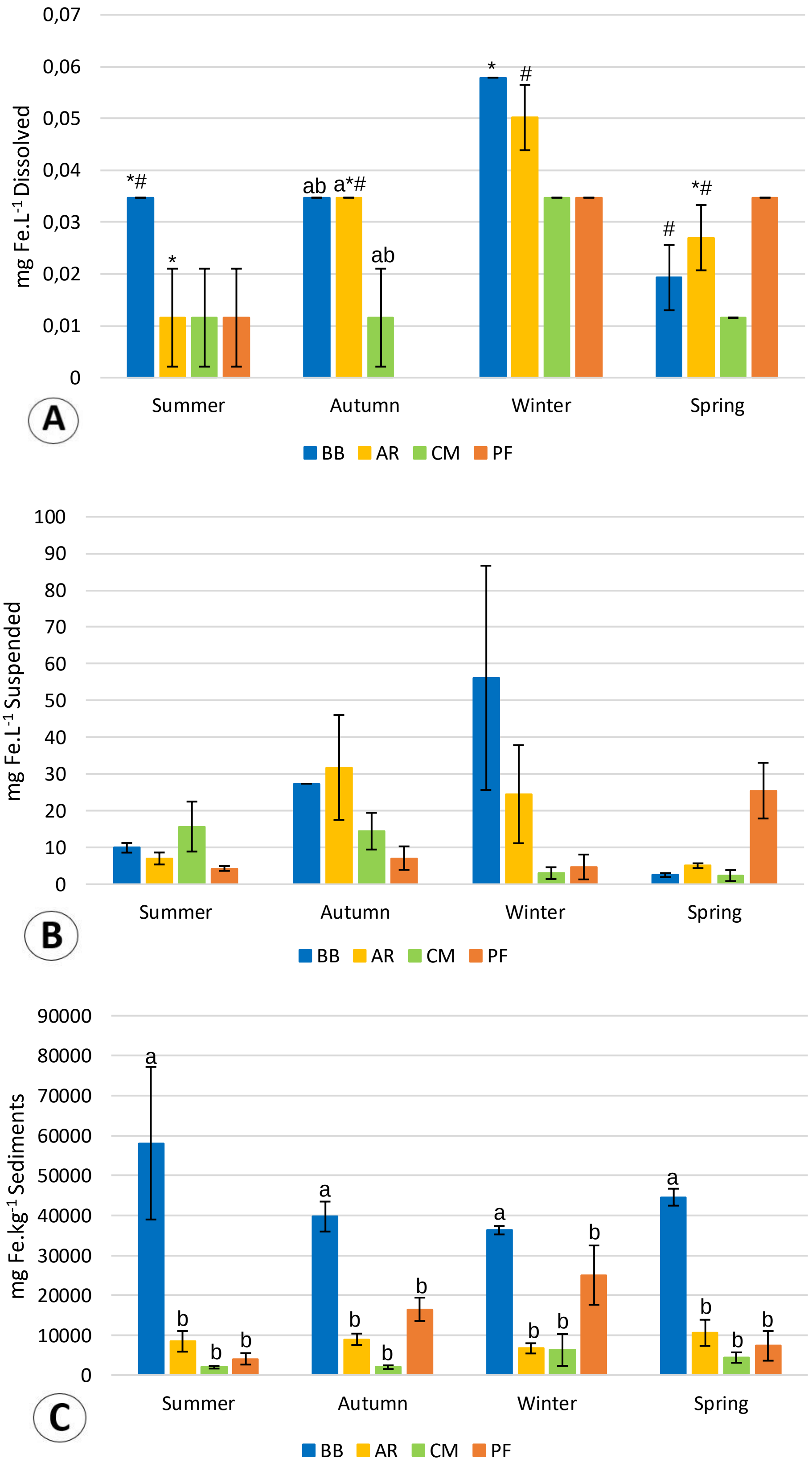

Figure 3.5 – Concentrations of Iron (Fe) in the environmental samples at the Óbidos Lagoon during the study period. (A) total dissolved Fe in the water column, in miligrams per litre; (B) total suspended Fe in the water column, in miligrams per litre (N=3, except for autumn at BB station); and (C) total Fe in sediments, in miligrams per kilogram (N=3, for each season and station). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (* and #) indicate significant differences between seasons in the different stations and the letters (a and b) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni and Tukey HSD tests); absence indicates that no significant differences were observed.

Manganese – Mn

The element manganese (Mn) was found in the suspended fraction of the water samples from all stations and seasons, while dissolved Mn was found in all seasons but only at two stations. In the sediment Mn was detected in all seasons and stations except once.

Manganese dissolved in the water column was detected in all seasons, however only at BB and AR stations. At BB station, it was not possible to detect dissolved Mn in autumn and spring, also the lowest concentration was detected in the winter (0.0018 mg.L^{-1} ± 0.0015, **Figure 3.6A**). Comparing with the other seasons, the highest concentration of dissolved manganese (Mn) in the water column was detected in the summer at BB (1.0000 mg.L^{-1} ± 0.1282, **Figure 3.6A**). The Two-Way ANOVA revealed effects between seasons and stations in the concentrations of dissolved Mn (ρ-value – 0.000, **Attachment 2**), with significant differences between the summer and the other three seasons at BB (Bonferroni test: ρ-values – 0.000, **Attachment 2**). It was also possible to find dissolved manganese at AR station in all seasons, though the values were lower than at BB in the summer (**Figure 3.6A**). The post-hoc test corroborates this evidence showing significant differences in the summer at BB station between BB and the other three stations (Bonferroni test: ρ-values – 0.000, **Attachment 2**).

The manganese suspended in the water column was detected in all seasons and sampling stations. Suspended manganese was found in higher concentration in the summer at BB station (4.0457 mg.L^{-1} ± 0.5704), followed by winter also at BB (3.4593 mg.L^{-1} ± 1.5414). In addition, at AR station concentrations were relatively higher compared with CM and PF stations, where suspended manganese was found in lower and similar concentrations in all seasons (**Figure 3.6B**). Those evidences were supported by the Two-Way ANOVA results, which revealed an effect of stations in suspended Mn (ρ-value – 0.000, **Attachment 3**), with the concentrations at station BB being significantly different from the stations CM and PF (Tukey HSD tests: ρ-value – 0.007 and ρ-value – 0.005, respectively, **Attachment 3**).

It was possible to detect manganese in the sediment samples in all seasons and stations, except for CM station in the summer. In all seasons, it is notable that higher concentrations of Mn in the sediments were found at BB station, with a range between 411.4867 mg.kg^{-1} ± 72.8983 and 926.4752 mg.kg^{-1} ± 279.1183. At AR and PF stations it was possible to detect relatively higher concentrations of manganese in the sediment when compared with CM station (**Figure 3.6C**). However, the difference between Mn in the sediment at BB station and the other stations is clear, and supported by the Two-Way ANOVA results which revealed an effect of stations in Mn concentrations in the sediment (ρ-value – 0.000, **Attachment 4**), with significant differences between BB station and the other three sampling stations (Tukey HSD test: ρ-values – 0.000, **Attachment 4**).

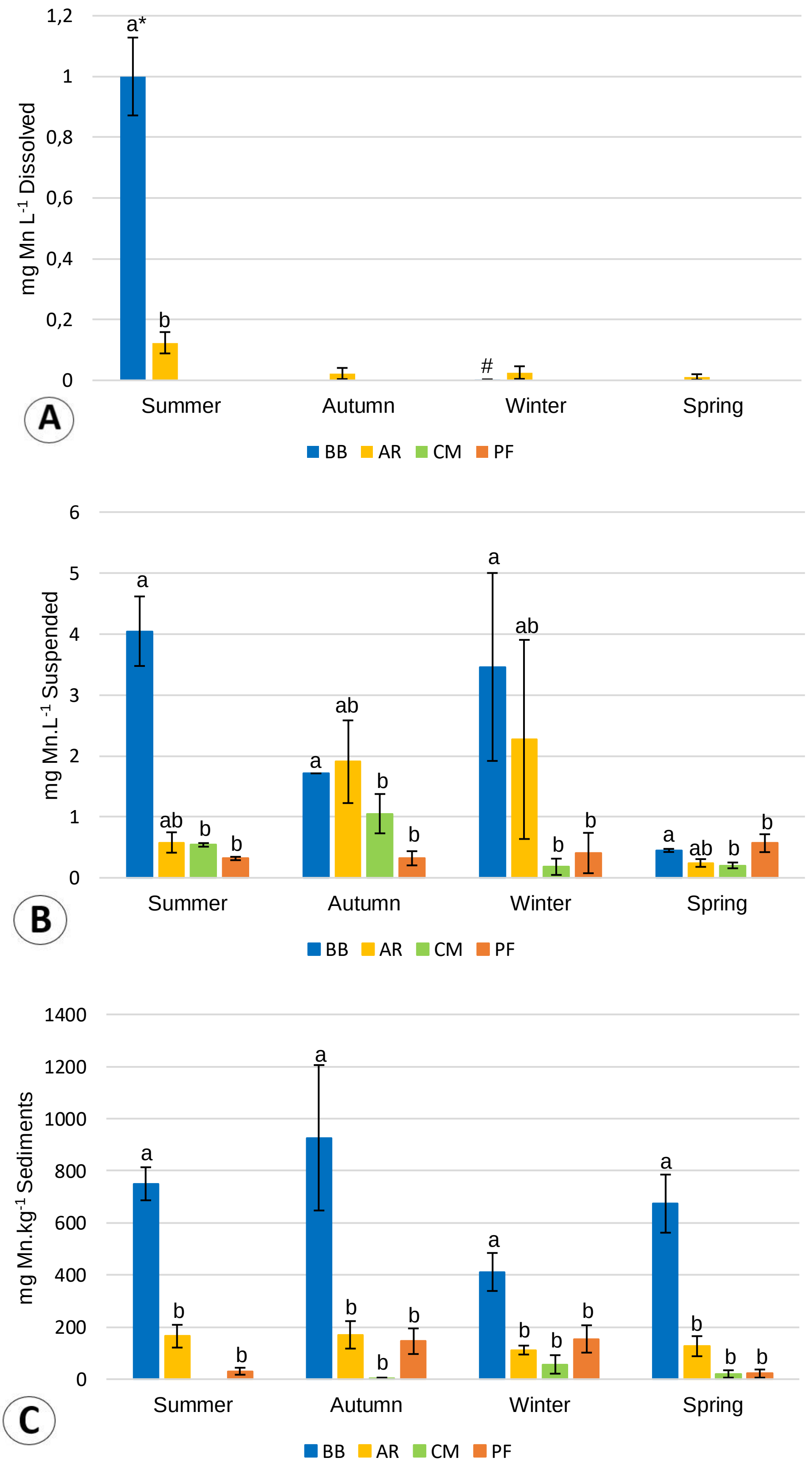

Figure 3.6 – Concentrations of Manganese (Mn) in the environmental samples at the Óbidos Lagoon during the studied period. (A) total dissolved Mn in the water column, in miligrams per litre (N=3, except for autumn at BB station); (B) total suspended Mn in the water column, in miligrams per litre (N=3, except for autumn at BB station) and (C) total Mn in sediments, in miligrams per kilogram (N=3, for each season and station). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (* and #) indicate significant differences between seasons in the different stations and the letters (a and b) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni and Tukey HSD tests); absence indicates that no significant differences were observed.

Zinc – Zn

Zinc was not detected in the dissolved fraction of the water samples, while suspended zinc was only found in the winter and spring in some stations. In the sediment Zn was detected in all seasons, however it was not possible to detect it in all stations.

Suspended zinc was found in the winter at BB and AR stations, and in the spring at AR, CM and PF, always in low concentrations (**Figure 3.7A**). However, the Two-Way ANOVA test did not show any statistical significant differences between seasons and/or stations.

The element zinc was possible to detect in the sediment in all seasons, however not in all sampling sites, such as PF station in the summer, and at CM station in any season. Zinc concentrations in sediments at BB station were higher in all seasons comparing with the other stations. Also, at BB station highest concentrations of Zn were found in summer (111.720 mg.kg^{-1} ± 17.473) and spring (108.586 mg.kg^{-1} ± 2.744), and lowest concentrations in autumn (75.021 mg.kg^{-1} ± 10.856) and in winter (66.789 mg.kg^{-1} ± 3.23) (**Figure 3.7B**). The Two-Way ANOVA test revealed effects between seasons and stations in the Zn concentrations in the sediment (ρ-value – 0.002, **Attachment 4**). Also, the post-hoc test results corroborates this tendency showing statistically significant differences at BB station, between summer and autumn (Bonferroni test: ρ-value of 0.012, **Attachment 4**); summer and winter (Bonferroni test: ρ-value of 0.001, **Attachment 4**); autumn and spring (Bonferroni test: ρ-value of 0.025, **Attachment 4**) and winter and spring (Bonferroni test: ρ-value of 0.003, **Attachment 4**). Additionally, the Zn concentration in the sediment at AR station was higher in the summer (14.411 mg.kg^{-1} ± 6.450) and in the three followed seasons, Zn concentration ranged between 4.045 mg.kg^{-1} ± 3.303 and 4.238 mg.kg^{-1} ± 3.460. Also, Zn concentrations in the sediment at PF station ranged between 3.238 mg.kg^{-1} ± 2.644 and 31.796 mg.kg^{-1} ± 7.700, with the highest concentration found in autumn and with a tendency to decrease in the next seasons (**Figure 3.7B**). As expected, the post-hoc tests results showed statistically significant differences between BB station and the other stations in all seasons with ρ-values of 0.000, with exception for BB and PF station, in the autumn (Bonferroni test: ρ-value of 0.002, **Attachment 4**). In this season, were also found statistically significant differences between CM and PF stations (Bonferroni test: ρ-value – 0.038, **Attachment 4**). In addition, significant differences were found between summer and autumn at PF station (Bonferroni test: ρ-value – 0.038, **Attachment 4**).

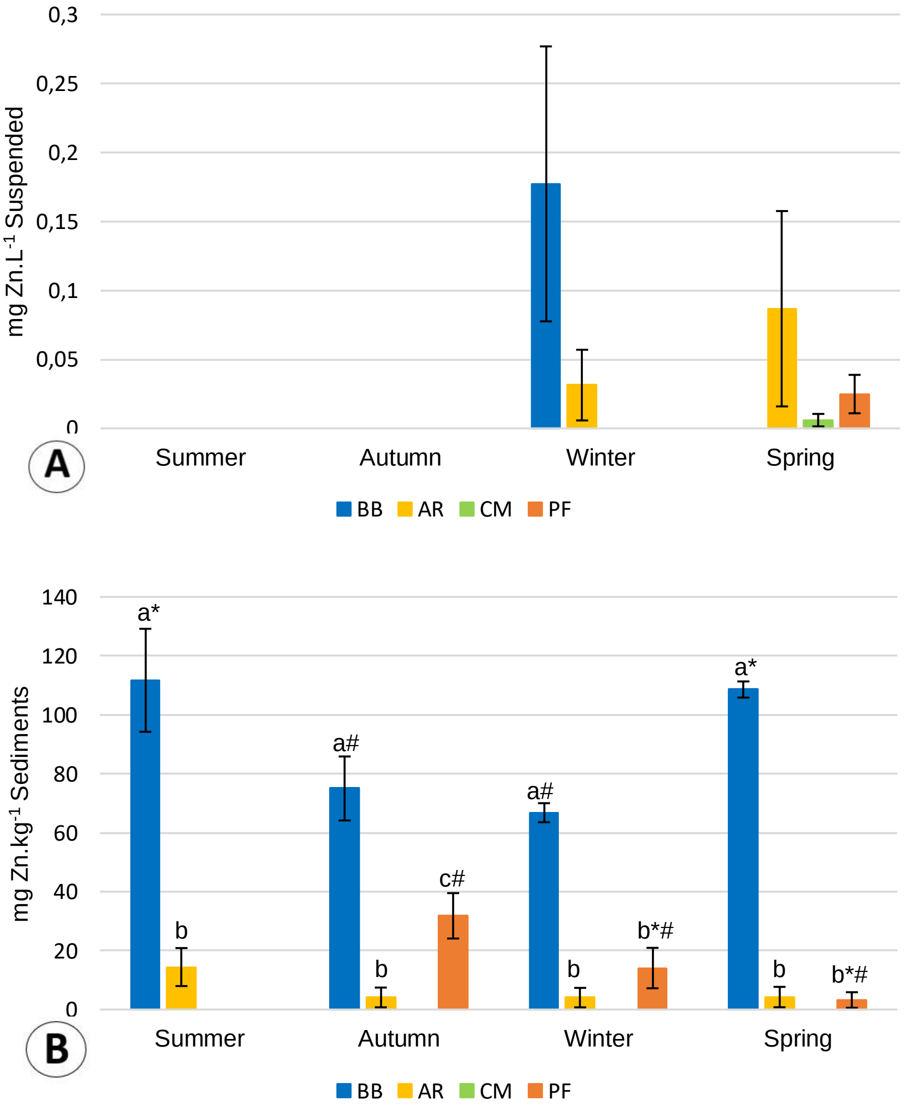

Figure 3.7 – Concentration of Zinc (Zn) in the environmental samples at the Óbidos Lagoon during the studied period. (A) total suspended Zn in the water column, in miligrams per litter (N=3, except for autumn at BB station); and (B) total Zn in sediments, in miligrams per kilogram (N=3, for each season and station). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (* and #) indicate significant differences between seasons in the different stations and the letters (a,b and c) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni test); absence indicates that no significant differences were observed.

Cadmium – Cd

The element Cd was detected in both sediment and water samples, however it was not possible to detect this element in all stations and seasons, especially in the sediments.

The dissolved Cd was detected in all seasons and stations, except for CM and PF stations in spring. The highest concentration was found at BB station, where the concentrations increased along the study period from 0.011 μg.L^{-1} ± 0.004 in summer 18 to 0.131 μg.L^{-1} ± 0.072 in spring 19. The lowest concentration was detected in summer at PF station (0.001 μg.L^{-1} ± 0.000), while in the autumn and winter similar concentrations were observed at this study site (between 0.026 μg.L^{-1} ± 0.010 and 0.030 μg.L^{-1} ± 0.012, respectively) (**Figure 3.8A**). However, Two-Way ANOVA results did not show any statistical significant differences between seasons and/or stations.

Regarding suspended Cd, in summer it was only possible to detect this metal at BB (0.015 μg.L^{-1} ± 0.013) and AR (0.009 μg.L^{-1} ± 0.007) stations and in small amounts. In autumn it was only detected at PF station, also in small amounts (0.009 μg.L^{-1} ± 0.000), while in winter it was only detected at BB station (0.678 μg.L^{-1} ± 0.553) and in spring only at AR station (0.415 μg.L^{-1} ± 0.339). In all seasons, suspended Cd was not detected at CM station. All stations tended to have low and similar suspended Cd concentrations (**Figure 3.8B**) and the statistical test results did not show any significant differences between seasons, stations or an interaction between both factors.

In sediment samples it was possible to find Cd in all studied seasons, except for autumn, and at all stations except AR. Also, in CM station Cd was only found in summer in low concentrations. The highest Cd concentration in sediment at BB station was detected in spring (59.7508 mg.kg^{-1} ± 48.7863), comparing with summer (0.0418 mg.kg^{-1} ± 0.0341) and winter (0.0002 mg.kg^{-1} ± 0.0002). In the PF station was only detected Cd in winter and spring, with winter characterized by a low concentration (0.0002 mg.kg^{-1} ± 0.0002), in contrast to spring (143.6561 mg.kg^{-1} ± 117.2947), which presented the highest concentration reported in this study (**Table 3.1**). Despite this, statistically significant differences between seasons and/or stations were not obtained.

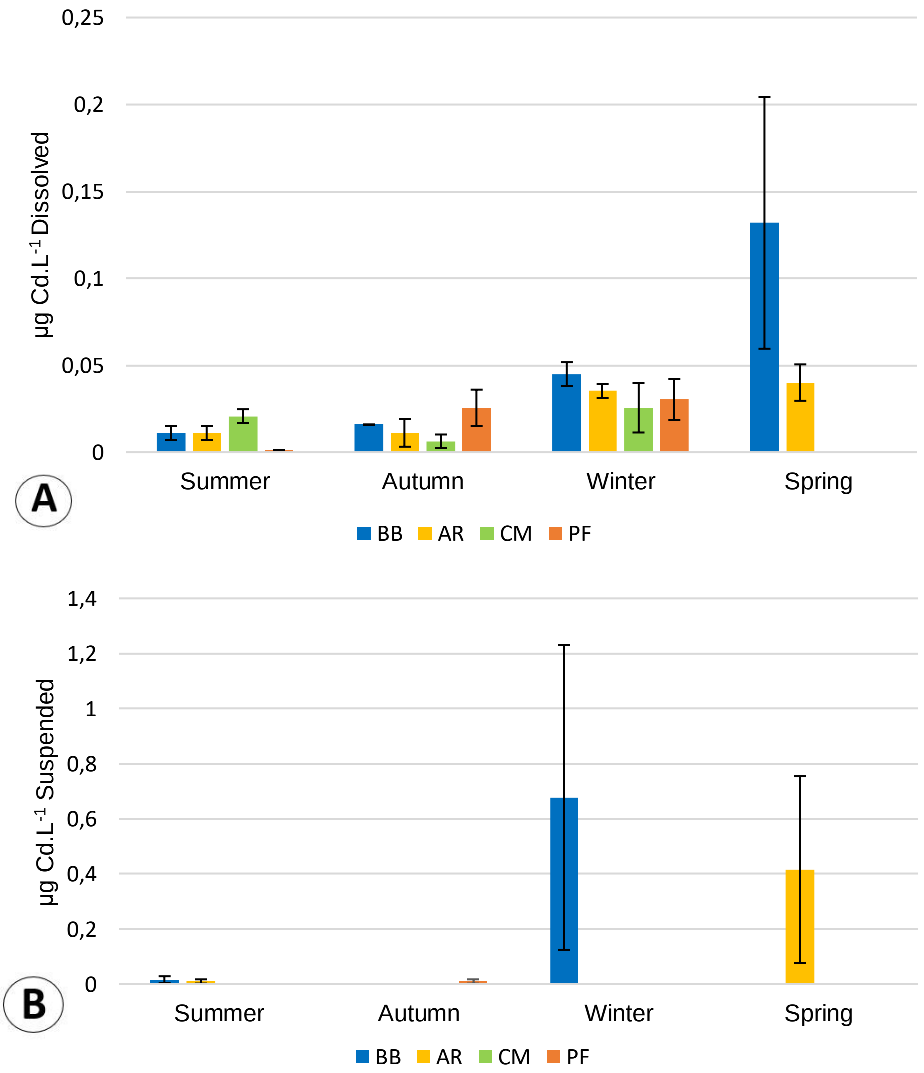

Figure 3.8 – Concentrations of Cadmium (Cd) in the environmental samples at the Óbidos Lagoon during the studied period. (A) total dissolved Cd in the water column, in micrograms per litter (N=3, except for autumn at BB station); and (B) total suspended Cd in the water column, in micrograms per litter (N=3, except for autumn at BB station). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF).

Table 3.1 – Concentrations of Cadmium (Cd) in the sediment samples at the Óbidos Lagoon during the studied period. Values were expressed in miligrams per kilogram (N=3, for each station and season); All values were expressed as mean ± standard deviation Sampling sites: *Barrosa's* Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). Limit of dectetion in micrograms per litre for Cd: 0.001.

Cd$_{Sediment}$ (mg.kg^{-1})				
	Summer	**Autumn**	**Winter**	**Spring**
BB	0.0418 ± 0.0341	<0.001	0.0002 ± 0.0002	59.7508 ± 48.7863
AR	<0.001	<0.001	<0.001	<0.001
CM	0.0733 ± 0.0598	<0.001	<0.001	<0.001
PF	<0.001	<0.001	0.0002 ± 0.0002	143.6561 ± 117.2947

Lead – Pb

The trace metal lead was not detected in the water samples in the dissolved form, but in suspension it was detected in all seasons, except spring, and in most stations. In the sediment samples it was possible to detect the presence of lead in all seasons and stations.

It was not possible to detect Pb in suspended matter in spring, and in summer only small concentrations were found at BB (0.936 $\mu g.L^{-1}$ ± 0.398) and PF (0.569 $\mu g.L^{-1}$ ± 0.465) stations. In the autumn, it was possible to detect suspended Pb at AR (4.6575 $\mu g.L^{-1}$ ± 2.1304), PF (4.0027 $\mu g.L^{-1}$ ± 3.2682) and CM (0.9457 $\mu g.L^{-1}$ ± 0.7721) stations. The highest Pb concentrations in suspended metals were found in the winter at all stations, except for PF station (**Figure 3.9A**). According to the Two-Way ANOVA results seasons had a statistical significant effect on suspended Pb (ρ-value of 0.002, **Attachment 3**), with statistically significant differences found between summer and winter (Tukey HSD test: ρ-value of 0.006, **Attachment 3**); autumn and winter (Tukey HSD test: ρ-value of 0.030, **Attachment 3**); and winter and spring (Tukey HSD test: ρ-value of 0.004, **Attachment 3**).

In the sediments Pb was detected in all stations and seasons. The highest Pb concentration was detected in summer at BB station (77.144 $mg.kg^{-1}$ ± 2.703) compared with the other seasons and stations. Also, at the other stations in the summer, the concentrations were smaller, with values between 11.205 $mg.kg^{-1}$ ± 1.298 and 3.126 $mg.kg^{-1}$ ± 1.080 (**Figure 3.9B**). The Two-Way ANOVA results revealed effects between seasons and stations in Pb concentrations in the sediment (ρ-value – 0.000, **Attachment 4**). In addition, the post-hoc test results showed significant differences between BB station and the other three stations in the summer (Bonferroni test: ρ-values – 0.000, **Attachment 4**). In the autumn and spring all the stations presented lower concentrations compared with the other seasons. In the winter, the concentrations at AR, CM and PF stations were higher than in the summer, autumn and spring (**Figure 3.9B**). The post-hoc test result showed significant differences at BB station between summer and the other seasons (Bonferroni test: ρ-value – 0.000, **Attachment 4**) and between autumn and winter (Bonferroni test: ρ-value – 0.049, **Attachment 4**); also at AR, CM and PF stations were found statistically significant differences between autumn and winter (ρ-values of 0.001, 0.004 and 0.002, respectively, **Attachment 4**). Additionally, at PF station were found statistically significant differences between summer and winter (Bonferroni test: ρ-value – 0.000, **Attachment 4**) and winter and spring (Bonferroni test: ρ-value – 0.000, **Attachment 4**).

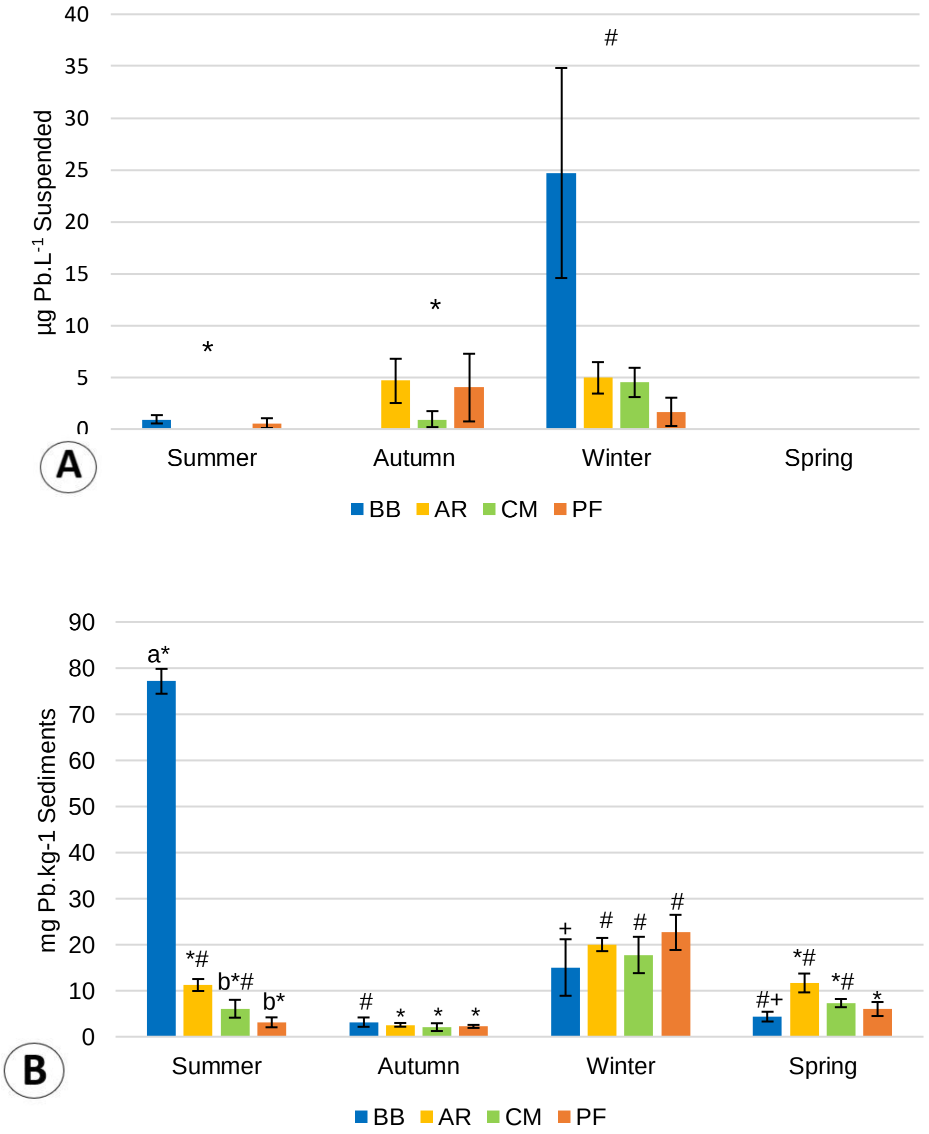

Figure 3.9 – Concentrations of Lead (Pb) in the environmental samples at the Óbidos Lagoon during the studied period. (A) total suspended Pb in the water column, in micrograms per litter (N=3, except for autumn at BB station); and (B) total Pb in the sediment, in micrograms per gram (N=3, for each season and station). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (* and #) indicate significant differences between seasons in the different stations and the letters (a and b) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Tukey HSD and Bonferroni tests); absence indicates that no significant differences were observed.

3.2.2 Biological samples – *Hediste diversicolor*

Despite all the efforts, it was not possible to collect *H. diversicolor* in all stations and seasons. In summer, due to unfavorable tide conditions, it was only possible to collect 7 organisms at AR and CM stations. Also, in winter the organisms were not collected at PF station since the tidal level did not go down far enough to reach the animals.

All studied metals were found in the organisms from all sampling sites and almost in all seasons. The essential metals were found in higher concentrations compared with non-essential metals and can be ranged by the following order: Fe>Zn>Mn>Cu>Pb>Cd. In general, essential metals were found in higher concentrations at AR and PF stations, while non-essential metals were found in higher concentrations at AR and BB stations.

The element copper was possible to detect in *H. diversicolor* in all studied seasons and sampling sites, except for BB station in the winter. The highest Cu concentrations were found at AR station (47.051 mg.kg^{-1} ± 8.231) and the lowest were found at CM (0.302 mg.kg^{-1} ± 0.280) (**Table 3.2**). The Two-Way ANOVA test revealed effects between seasons and stations in the Cu concentrations in the organisms (ρ-value – 0.004, **Attachment 5**). The post-hoc test results supported this showing significant differences between the AR and CM stations in the summer and autumn (ρ-values – 0.000, **Attachment 5**). Also, in the spring it was possible to find Cu in the organisms at all stations, in similar and low concentrations (**Table 3.2**). In addition, the post-hoc test results showed statistically significant differences at AR stations between summer and autumn (Bonferroni test: ρ-value – 0.044, **Attachment 5**); summer and winter (Bonferroni test: ρ-value – 0.000, **Attachment 5**); summer and spring (Bonferroni test: ρ-value – 0.000, **Attachment 5**); and autumn and spring (Bonferroni test: ρ-value – 0.013, **Attachment 5**).

The metal iron was detected in *H. diversicolor* in all sampling sites during all the study periods. This element was found in highest concentrations in the autumn at BB (562 mg.kg^{-1} ± 61) and in the winter at CM station (772 mg.kg^{-1} ± 370). Despite this, all stations showed lower and similar concentrations in the other seasons (**Table 3.2**) and the two-way ANOVA test results did not show any significant differences between seasons or stations and, consequently, any effects were observed in Fe concentrations in the organisms.

The element manganese was not detected in the organisms in summer at none of the sampling sites. Also, in the two followed seasons (autumn and winter), Mn was not detected at all stations, and when it was, it was only found in small amounts. On the contrary, the spring was characterized by higher concentrations of manganese in the organisms at all stations (**Table 3.2**). The Two-Way ANOVA test showed effects between seasons and stations in Mn concentrations in the organisms (ρ-value – 0.000, **Attachment 5**). Significant differences were observed at all stations between the spring and the other three seasons (Bonferroni test: ρ-values – 0.000, **Attachment 5**). Also, in the spring higher values were found at AR, followed by PF station and post-hoc test supported this showing significant differences in the spring between AR and the other three stations (Bonferroni test: ρ-values – 0.000, **Attachment 5**) and between CM and PF station (Bonferroni test: ρ-value – 0.033, **Attachment 5**).

The element zinc was found in the organisms from all the sampling sites and studied seasons. The highest concentration in the organisms was detected at AR station in the winter (133.195 mg.kg^{-1} ± 24.021) and the lowest concentration was detected at CM station in the summer (52.725 mg.kg^{-1} ± 13.239). In all seasons zinc was found in similar concentrations at all stations, except at BB station where higher Zn concentrations were detected in autumn (122.558 mg.kg^{-1} ± 8.645) (**Table 3.2**). The Two-Way ANOVA test revealed effects between seasons and stations in Zn concentrations in the organisms (ρ-value – 0.002, **Attachment 5**). The post-hoc test confirmed this showing significant differences at BB station between autumn and winter (Bonferroni test: ρ-value – 0.024, **Attachment 5**). In the summer, the AR station (116.1525 mg.kg^{-1}) was characterized by highest concentrations than the CM station (**Table 3.2**) and the post-hoc test results confirmed the presence of statistically significant differences between AR and CM stations in summer (Bonferroni test: ρ-value – 0.041, **Attachment 5**). In the autumn, the highest Zn concentration was found at PF station and the lowest at AR station (57.107 mg.kg^{-1} ± 16.550 and 129.332 mg.kg^{-1} ± 8.266). The post-hoc test results showed statistically significant differences between AR and PF stations in the autumn (Bonferroni test: ρ-value – 0.034, **Attachment 5**). In the winter, the highest concentration of Zn in the organisms was found at AR station (121.266 mg.kg^{-1} ± 24.021) and the lowest at BB station (**Table 3.2**), also the post-hoc test results showed significant differences between these stations (Bonferroni test: ρ-value of 0.007, **Attachment 5**). In the spring the Zn concentrations were similar at all stations (**Table 3.2**) and no significant differences were found.

The highest Cd concentrations in *H. diversicolor* were found at AR station in all the studied seasons. At BB station the highest Cd concentrations in the organisms were found in winter (0.312 $mg.kg^{-1}$ ± 0.144), while the lowest concentrations were found in autumn (**Table 3.2**). The Two-Way ANOVA test only revealed effects between stations in the Cd concentrations in the organisms (ρ-value – 0.002, **Attachment 5**). At CM and PF stations the Cd concentrations in the organisms were lower compared with AR station and the post-hoc test results showed significant differences between AR and CM stations (Bonferroni test: ρ-value – 0.000, **Attachment 5**) and AR and PF stations (Bonferroni test: ρ-value – 0.004, **Attachment 5**).

The element lead was found in the organisms from all stations and periods of study. The highest concentrations were found in the autumn at AR station (8.033 $mg.kg^{-1}$ ± 2.094) and the lowest concentrations were found at CM station also in the autumn (0.061 $mg.kg^{-1}$ ± 0.055). The summer was characterized by similar Pb concentrations at AR (2.436 $mg.kg^{-1}$ ± 0.898) and CM (2.154 $mg.kg^{-1}$ ± 0.825) stations (**Table 3.2**). With the exception of autumn at AR station, the Pb concentrations in the other three stations were lower and similar. The Two-Way ANOVA test revealed effects between seasons and stations in Pb concentrations in the organisms (ρ-value – 0.049, **Attachment 5**). The post-hoc test results confirmed this showing significant differences in this season, between BB and AR stations (Bonferroni test: ρ-value – 0.036, **Attachment 5**); AR and CM stations (Bonferroni test: ρ-value – 0.005, **Attachment 5**); and AR and PF stations (Bonferroni test: ρ-value – 0.005, **Attachment 5**). In the winter, the highest Pb concentrations were found at BB station (7.074 $mg.kg^{-1}$ ± 4.569), and the lowest concentrations were found at CM station (0.499 $mg.kg^{-1}$ ± 0.249) (**Table 3.2**). To support this, the post-hoc tests results showed significant differences between BB and CM stations in the winter (Bonferroni test: ρ-value – 0.017, **Attachment 5**). In the spring, similar and lower concentrations were found at CM and PF stations, and the highest Pb concentration was found at AR station (3.785 $mg.kg^{-1}$ ± 1.645). In addition at BB station, the concentrations were higher (1.098 $mg.kg^{-1}$ ± 0.384) (**Table 3.2**), however lower than winter and the post-hoc test results showed significant differences at BB station between winter and spring (Bonferroni test: ρ-value – 0.034, **Attachment 5**).

Table 3.2 – Concentrations of Copper (Cu), Iron (Fe), Manganese (Mn), Zinc (Zn), Cadmium (Cd) and Lead (Pb) in *H. diversicolor* organisms collected at Óbidos Lagoon during the studied period. All values were expressed as mean ± standard error (N=7 in summer at AR and CM stations; N=10, in the autumn and spring at all stations and in the winter at BB, AR and CM stations). Sampling sites: *Barrosa'*s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). Limits of detection in micrograms per litre: 0.4 for Cu; 0.035 for Mn; 0.001 for Cd. The symbols (*, # and +) indicate significant differences between seasons in the different stations and the letters (a, b and c) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni and Tukey HSD tests); absence indicates that no significant differences were observed.

		Essentials metals				**Nonessentials metals**	
Seasons	**Stations**	**Cu (mg.kg^{-1})**	**Fe (mg.kg^{-1})**	**Mn (mg.kg^{-1})**	**Zn (mg.kg^{-1})**	**Cd (mg.kg^{-1})**	**Pb (mg.kg^{-1})**
Summer	**BB**	–	–	–	–	–	–
	AR	47.051 ± 8.231 **a***	478.136 ± 52.777	<0.035	116.152 ± 30.402 **a**	0.133 ± 0.067	2.436 ± 0.898 **a**
	CM	0.302 ± 0.280 **b**	382.857 ± 32.796	<0.035	52.725 ± 13.239 **b**	0.120 ± 0.055	2.154 ± 0.825 **b**
	PF	–	–	–	–	–	–
Autumn	**BB**	6.415 ± 4.035	561.821 ± 60.769	<0.035	122.558 ± 8.645 *****	0.062 ± 0.042	1.520 ± 0.832 **a*#**
	AR	24.459 ± 10.468 **a#**	204.870 ± 54.518	<0.035	57.107 ± 16.550 **b**	0.335 ± 0.087	8.033 ± 2.094 **b**
	CM	0.333 ± 0.134 **b**	288.408 ± 25.111	2.499 ± 2.324 *****	93.749 ± 9.139	0.001 ± 0.0006	0.061 ± 0.055 **a**
	PF	11.950 ± 3.982	359.966 ± 17.273	3.089 ± 2.651 *****	129.332 ± 8.266 **a**	0.051 ± 0.011	0.066 ± 0.048 **a**
Winter	**BB**	<0.4	382.203 ± 58.907	<0.035	53.221 ± 17.113 **b#**	0.312 ± 0.144	7.074 ± 4.569 *****
	AR	11.987 ± 11.174 **#+**	239.987 ± 58.328	<0.035	133.195 ± 24.021 **a**	0.449 ± 0.173	3.543 ± 0.584
	CM	4.414 ± 2.737	771.776 ± 370.021	2.759 ± 2.271 *****	95.464 ± 7.208	0.013 ± 0.012	0.499 ± 0.249
	PF	–	–	–	–	–	–
Spring	**BB**	2.333 ± 1.590	336.251 ± 46.180	33.515 ± 6.226 **ac**	72.312 ± 14.564 ***#**	0.099 ± 0.066	1.098 ± 0.383 **#**
	AR	0.862 ± 0.818 **+**	410.415 ± 32.317	66.225 ± 4.809 **b**	121.266 ± 35.005	0.384 ± 0.229	3.785 ± 1.645
	CM	1.988 ± 0.743	435.537 ± 24.530	32.021 ± 3.586 **c#**	84.247 ± 4.552	0.005 ± 0.005	0.198 ± 0.100
	PF	1.813 ± 1.128	471.155 ± 26.428	44.421 ± 3.798 **a#**	90.360 ± 15.005	<0.001	0.135 ± 0.055

3.3 Characterization of the biological responses

As previously mentioned, it was not possible to collect *H. diversicolor* in all stations and seasons. The tide conditions were not favorable in the summer, allowing only the collection of 7 organisms at AR and CM stations, while in winter it was not possible to collect organisms at PF station.

3.3.1 Biometrical characterization and Sex Determination

The measurements of L3 of *H. diversicolor* at the sampled stations of Óbidos Lagoon during the study period ranged between 1.551 mm ± 0.059 at BB station in autumn and 2.993 mm ± 0.097 at CM station in spring. Also, in spring, were obtained the highest values at all stations comparing with the other seasons (**Figure 3.10A**). The Two-Way ANOVA test revealed effects between seasons and stations in L3 measurements of the organisms (ρ-value – 0.001, **Attachment 6**). Also, the post-hoc test supported this showing significant differences between spring and all seasons at all stations (Bonferroni test: ρ-values – 0.000 and ρ-value – 0.007, **Attachment 6**). In addition, comparing with CM and PF stations L3 values were significantly lower at BB and AR stations in all seasons, except for summer (Bonferroni test: ρ-values between 0.000 and 0.032, **Attachment 6**).

The animals presented the highest wet weight (WW) values at CM station in spring (962 mg ± 54) and the lowest at BB station in autumn (118 mg ± 14). In general, at all seasons higher WW values were found at CM and PF stations compared with BB and AR stations (**Figure 3.10B**). The Two-Way ANOVA test revealed effects between seasons and stations in the wet weight of the organisms (ρ-value – 0.000, **Attachment 6**). In the autumn, significant differences on the WW of the animals between BB station and the other three stations were observed (Bonferroni test: ρ-values – 0.000). Additionally, statistically significant differences between AR and PF stations (Bonferroni test: ρ-value – 0.000) and at CM and PF stations (Bonferroni test: ρ-value – 0.005) were also found in the autumn (**Attachment 6**). The winter was characterized by highest wet weights at CM station (811 mg ± 48) (**Figure 3.10B**), which were significantly different from the WW's obtained at BB and AR stations (Bonferroni test: ρ-values – 0.000, **Attachment 6**). In the spring, the highest wet weights were also found at CM station, followed by PF (420 mg ± 41) (**Figure 3.10B**), and the post-hoc test results showed significant differences in the spring between all sampling stations (Bonferroni test results: BB and AR ρ-value – 0.012; BB and CM ρ-value – 0.000; BB and PF ρ-value – 0.021; AR and CM ρ-value – 0.000; AR and PF ρ-value – 0.000; CM and PF ρ-value – 0.000, **Attachment 6**). As mentioned before, at CM station the organisms presented higher wet weights in the spring and winter (**Figure 3.10B**) and the post-hoc test results showed significant differences between spring and all the other seasons (Bonferroni test results: summer and spring ρ-value – 0.000; autumn and spring ρ-value – 0.000; winter and spring ρ-value – 0.002, **Attachment 6**). Additionally, were found statistically significant differences between winter and the seasons summer and autumn (Bonferroni test: ρ-values – 0.000, **Attachment 6**).

The biggest body sizes of *H. diversicolor* specimens were observed in winter at CM station (125 mm ± 6), while the smallest body sizes were found in autumn at BB station (40 mm ± 2). Also, summer and autumn were characterized by lower body sizes at all sampling stations, except for PF (**Figure 3.10C**). The Two-Way ANOVA test revealed effects between seasons and stations in body size of the organisms (ρ-value – 0.000, **Attachment 6**), with significant differences at AR station between summer and winter (Bonferroni test: ρ-value – 0.022). Also, at BB station the post-hoc test results showed significant differences between seasons (Bonferroni test results: autumn and winter ρ-value – 0.000, autumn and spring ρ-value – 0.000; winter and spring ρ-value – 0.043, **Attachment 6**). In addition, at CM station, the post-hoc test results showed significant differences between seasons (Bonferroni test: summer and winter ρ-value – 0.000; summer and spring ρ-value – 0.000; autumn and winter ρ-value – 0.000; autumn and spring ρ-value – 0.000; and winter and spring ρ-value – 0.002, **Attachment 6**). In general, comparing with BB and AR stations, the bigger body sizes were found at CM, followed by PF station (**Table 3.10C**). and statistic confirmed this. Significant differences were found between organism's body size from BB station and the other stations in the autumn (Bonferroni test: ρ-values – 0.000, **Attachment 6**); and in the spring between BB and the other three stations (Bonferroni test results: BB and AR ρ-value – 0.012;BB and CM ρ-value – 0.000; BB and PF ρ-value – 0.000, **Attachment 6**) and in the winter only were detected between BB and CM in the winter (Bonferroni test: ρ-value – 0.000, **Attachment 6**). Also were found statistically significant differences between PF and AR stations in the autumn (Bonferroni test: ρ-value – 0.000, **Attachment 6**) and in the spring (Bonferroni test: ρ-value – 0.000, **Attachment 6**) and between CM station in the autumn (Bonferroni test: ρ-value – 0.005, **Attachment 6**) and spring (Bonferroni test: ρ-value – 0.001, **Attachment 6**).

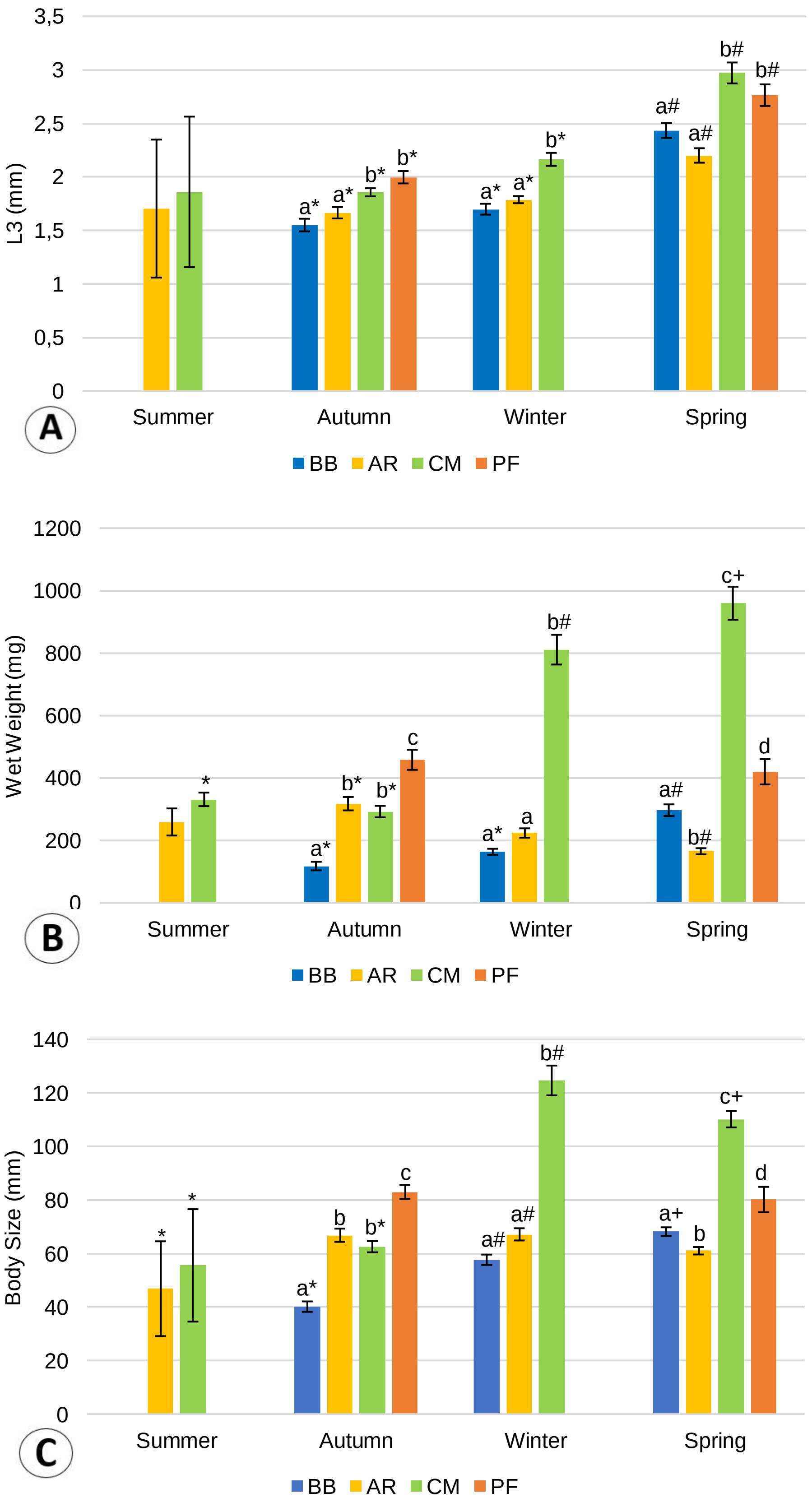

Figure 3.10 – Biometrical characterization of *H. diversicolor* collected at Óbidos Lagoon during the studied period. (A) peristomium, prostomium and first chaetiger length (L3) in milimeters (N=7 in summer; N=30 in the autumn, winter and at PF stations in the spring) N=29 at BB and AR stations in spring; N=27 at CM station in the spring); (B) wet weight in miligrams (N=7 in summer and N=30 in autumn, winter and spring); and (C) and body size in mm (N=7 in summer and N=30 in autumn, winter and spring). All values were expressed as mean and standard error was expressed in error bars. Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). The symbols (*, # and +) indicate significant differences between seasons in the different stations and the letters (a, b, c and d) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni test); absence indicates that no significant differences were observed.

As previously mentioned, (see subsection 2.6.1), it was not possible to identify male organisms. At CM station it was clear a higher female ratio in all seasons, while at AR station this trend was only observed in the summer. In the other stations the female ratio was lower than 50% (**Table 3.3**). These results were expected due to the non-determinate organisms, which probably englobe immature females and males.

Table 3.3 – Sex determination of *Hediste diversicolor* collected at Óbidos Lagoon in the studied period. Where, F – female organisms; ND – non determinate; %F – percentage of female organisms. Sampling sites: *Barrosa'*s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF).

	SUMMER			**AUTUMN**			**WINTER**			**SPRING**		
	F	**ND**	**%F**	**F**	**ND**	**%F**	**F**	**ND**	**%F**	**F**	**ND**	**%F**
BB	–	–	–	7	23	**23**	9	21	**30**	15	15	**50**
AR	7	0	**100**	11	19	**37**	7	23	**23**	4	26	**13**
CM	4	3	**57**	17	13	**57**	27	3	**90**	26	4	**87**
PF	–	–	–	14	16	**47**	–	–	–	10	20	**33**

3.3.2 Lipids content

In all the studied seasons, except for spring, the lipid content of the animals tended to be higher at CM station than at AR station. For both stations, higher lipid contents were found in the autumn, followed by spring and summer (**Figure 3.11**) and the Mann-Whitney test confirmed this showing significant values between summer and autumn (Mann-Whitney test: ρ-value – 0.002); autumn and spring (Mann-Whitney test: ρ-value – 0.009); summer and spring (ρ-value – 0.043). The winter was characterized by lowest lipid content at both stations, comparing with the other seasons and statistic test supported this showing significant differences between autumn and winter (Mann-Whitney test: ρ-value – 0.004); and winter and spring (Mann-Whitney test: ρ-value – 0.031).

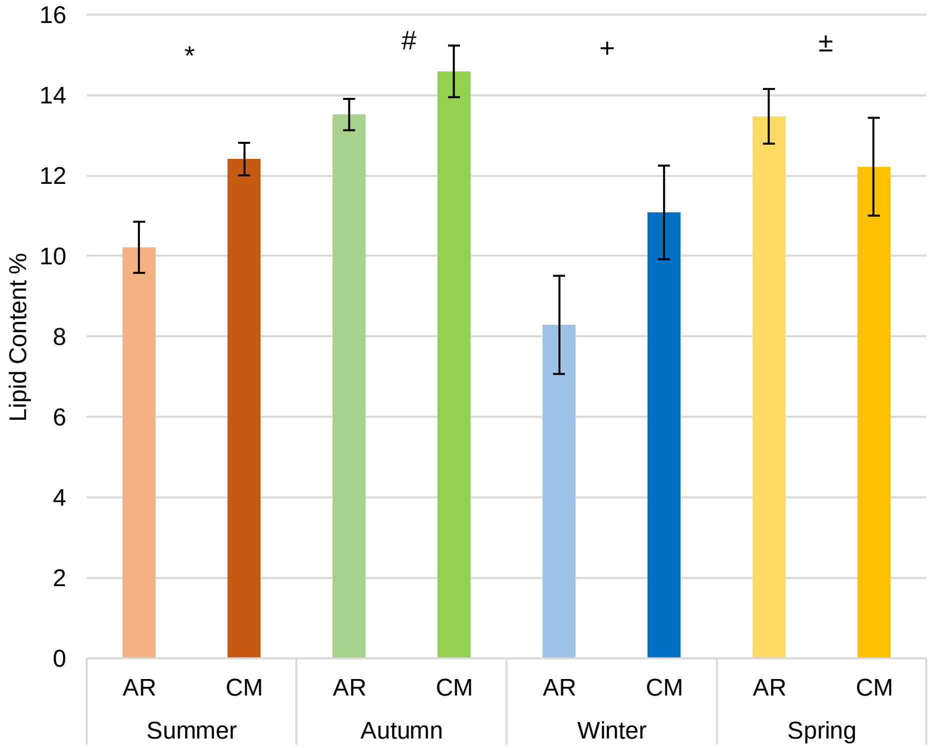

Figure 3.11 – Lipid content in % of *H. diversicolor* collected at Óbidos Lagoon during the study period. All values were expressed as mean and standard error was expressed in error bars (N=7). Sampling sites: *Arnóia* and *Real* rivers (AR) and *Covão dos Musaranhos* (CM). The symbols (*, #, + and ±) indicate significant differences between seasons, which correspond to ρ-value<0.050 (Mann-Whitney test).

3.3.3 Accumulation ability of *Hediste diversicolor* (BAF e CF)

For all the studied metals it was possible to calculate the bioaccumulation factors (BAF) in the organisms, but bioaccumulation was not observed for the element Fe (BAF<1) and in the other metals, the bioaccumulation did not occur in all seasons and sampling sites. In general, BAF values may be ranked in the following order in *H. diversicolor*: Cd>Zn>Cu>Pb>Mn>Fe (**Table 3.4**).

Bioaccumulation factors of *H. diversicolor* specimens for copper (Cu) were obtained in all seasons, although BAF values higher than 1 were only found at AR and CM stations. The higher BAF values were found in summer at AR station (7.736 ± 1.353) and the lowest BAF values were found at BB station in spring (0.059 ± 0.040). Also, in spring the animals were characterized by lower BAF values at AR station (**Table 3.4**). The Two-Way ANOVA test revealed effects between seasons and stations in Cu bioaccumulation in the organisms (ρ-value – 0.019, **Attachment 7**), with significantly higher values observed at AR station in summer as compared to the other seasons (Bonferroni test results: summer and autumn ρ-value – 0.032; summer and winter ρ-value – 0.031; summer and spring ρ-value – 0.000, **Attachment 7**). In addition, significant differences were also found between AR and CM stations in summer (Bonferroni test: ρ-value – 0.000, **Attachment 7**).

It was possible to obtain the iron (Fe) bioaccumulation factors (BAF) from the organisms in all studied seasons and stations. However, none of the values were above 1. Also, Fe BAF values of the animals at CM station were always higher than at the other stations, in all the studied seasons (**Table 3.4**). The Two-Way ANOVA revealed an effect of the sampling stations in Fe bioaccumulation in the organisms (ρ-value – 0.000, **Attachment 7**), with significant differences between CM station and the other three stations (Tukey HSD test: ρ-values – 0.000, **Attachment 7**).

Concerning the manganese bioaccumulation factor in *H. diversicolor,* it was not possible to calculate it in summer, at any station, in autumn and winter at BB and AR stations, and in winter at PF. The highest Mn BAF values were found in the animals from spring at all stations. On the other hand, Mn BAF values were above 1 exclusively in spring at CM and PF stations. The Two-Way ANOVA revealed effects between seasons and stations in Mn bioaccumulation factor in the organisms (ρ-value – 0.000, **Attachment 7**). Significant differences were obtained at CM station between summer and spring (Bonferroni test: ρ-value – 0.002, **Attachment 7**); and at PF station between autumn and spring (Bonferroni test: ρ-value – 0.002, **Attachment 7**). In the spring, the highest BAF values were found at PF (2.066 ± 0.177) and the lowest at BB station (0.050 ± 0.009) (**Table 3.4**). In fact, significant differences between BB and PF stations were detected in spring (Bonferroni test: ρ-value – 0.000, **Attachment 7**), but also between BB and CM stations (Bonferroni test: ρ-value – 0.001, **Attachment 7**); AR and CM stations (Bonferroni test: ρ-value – 0.043, **Attachment 7**); and AR and PF stations (Bonferroni test: ρ-value – 0.001, **Attachment 7**).

The bioaccumulation factors (BAF) for the element zinc (Zn) were possible to calculate for the animals from all stations, except for CM station, and values above 1 were also found at all stations. The highest Zn BAF values were found in animals from AR station in winter (32.926 ± 5.938) and spring (28.615 ± 8.260) and from PF station in spring (27.908 ± 4.634) (**Table 3.4**). The animals at BB station were characterized by low BAF values when it was possible to calculate. The Two-Way ANOVA test revealed effects between seasons and stations in Zn bioaccumulation factor in the organisms (ρ-value – 0.000, **Attachment 7**), with significant differences at AR station between summer and winter (Bonferroni test: ρ-value – 0.000, **Attachment 7**), summer and spring (Bonferroni test: ρ-value – 0.002, **Attachment 7**), autumn and winter (Bonferroni test: ρ-value – 0.002, **Attachment 7**) and autumn and spring (Bonferroni test: ρ-value – 0.028, **Attachment 7**). Also, at PF station significant differences were found between autumn and spring (Bonferroni test: ρ-value – 0.000). In addition, significant differences were found in autumn between AR and CM stations (Bonferroni test: ρ-value – 0.044); in the winter, between BB and AR stations (Bonferroni test: ρ-value – 0.000), and AR and CM stations (Bonferroni test: ρ-value – 0.000); finally, in spring, were detected statistically significant differences between the different stations (Bonferroni test results: BB and AR stations ρ-value – 0.000; BB and PF stations ρ-value – 0.000); AR and CM stations ρ-value – 0.000); and CM and PF stations ρ-value – 0.000, **Attachment 7**).

The bioaccumulation factors (BAF) of *H. diversicolor* for cadmium (Cd) were only obtained at BB and CM stations in specific seasons and only one BAF value was below 1. The highest Cd BAF values were found in the winter at BB station (1617 ± 747) and in the spring the value was significantly lower (0.002 ± 0.001) (**Table 3.4**). The Two-Way ANOVA test revealed effects between seasons and stations in Cd bioaccumulation factor in the organisms (ρ-value – 0.002, **Attachment 7**). Significant differences were observed at BB station between autumn and winter (Bonferroni test: ρ-value – 0.000, **Attachment 7**) and winter and spring (Bonferroni test: ρ-value – 0.000, **Attachment 7**). The only significant BAF values at CM station were found in the summer (1.633 ± 0.745) and were greater lower than at BB station, however Cd BAF values at CM were not detected in the winter and the post-hoc test results showed significant differences in the winter between BB and AR stations (Bonferroni test: ρ-value – 0.000, **Attachment 7**) and between BB and CM stations (Bonferroni test: ρ-value – 0.000, **Attachment 7**).

The bioaccumulation factors (BAF) of *H. diversicolor* for lead (Pb) were obtained at all stations and in all seasons. However, BAF values above 1 were only obtained at AR station in autumn (3.120 ± 0.813, **Table 3.4**), while in the other seasons the BAF values were considerably lower. The Two-Way ANOVA revealed effects between seasons and stations in Pb bioaccumulation factor (ρ-value – 0.000, **Attachment 7**). At AR station significantly higher BAF Pb values were obtained in autumn as compared to the other seasons (Bonferroni test results: autumn and summer ρ-value – 0.000; autumn and winter ρ-value – 0.000; autumn and spring ρ-value – 0.000, **Attachment 7**). At the other sampling stations the BAF values were quite similar, and statistically significant differences were only found in the autumn between AR and the other three stations (Bonferroni test: ρ-values – 0.000, **Attachment 7**).

Table 3.4 – Bioaccumulation factors (BAF) of Copper (Cu), Iron (Fe), Manganese (Mn), Zinc (Zn), Cadmium (Cd) and Lead (Pb) in *Hediste diversicolor* collected at Óbidos Lagoon during the study period. All values were expressed as mean ± standard error (N=7 in the summer at AR and CM stations; N=10, in the autumn and spring at all stations, and in the winter at BB, AR and CM stations). Sampling sites: *Barrosa'*s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). BAF values represented in bold correspond to values above 1. Limits of detection in micrograms per litre: 0.4 for Cu, 0.035 for Mn; and 0.001 for Cd. The symbols (* and #) indicate significant differences between seasons in the different stations and the letters (a, b and c) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni test); absence indicates that no significant differences were observed.

Seasons/ Stations		Essentials metals				Nonessentials metals	
		BAF_Cu	**BAF_Fe**	**BAF_Mn**	**BAF_Zn**	**BAF_Cd**	**BAF_Pb**
Summer	**BB**	–	–	–	–	–	–
	AR	**7.736 ± 1.353** *	0.057 ± 0.006	<0.035	**8.060 ± 2.110** *	<0.001	0.217 ± 0.080 *
	CM	<0.4	0.195 ± 0.017	<0.035	na	**1.633 ± 0.745**	0.355 ± 0.136
	PF	–	–	–	–	–	–
Autumn	**BB**	0.204 ± 0.128	0.014 ± 0.002	<0.035	**1.634 ± 0.115**	<0.001	0.480 ± 0.262 **a**
	AR	**3.144 ± 1.345** #	0.023 ± 0.006	<0.035	**13.905 ± 4.030** a*	<0.001	**3.120 ± 0.813** **b#**
	CM	0.219 ± 0.088	0.145 ± 0.013	0.750 ± 0.697	na	<0.001	0.030 ± 0.026 **a**
	PF	0.637 ± 0.212	0.022 ± 0.001	0.021 ± 0.018 *	**4.068 ± 0.260** b*	<0.001	0.030 ± 0.027 **a**
Winter	**BB**	<0.4	0.011 ± 0.002	<0.035	0.797 ± 0.256 **a**	**1617 ± 747** *	0.471 ± 0.304
	AR	**3.117 ± 2.905** #	0.036 ± 0.009	<0.035	**32.926 ± 5.938** **b#**	<0.001	0.177 ± 0.029 *
	CM	**1.062 ± 0.659**	0.123 ± 0.059	0.651 ± 0.548	na	<0.001	0.028 ± 0.014
	PF	–	–	–	–	–	–
Spring	**BB**	0.059 ± 0.040	0.008 ± 0.001	0.050 ± 0.009 **a**	0.666 ± 0.134 **a**	0.002 ± 0.001 #	0.252 ± 0.088
	AR	0.099 ± 0.094	0.039 ± 0.003	0.523 ± 0.038 **c**	**28.615 ± 8.260** **b#**	<0.001	0.324 ± 0.141 *
	CM	**1.218 ± 0.455** #	0.099 ± 0.006	**1.595 ± 0.179** **b**	na	<0.001	0.027 ± 0.014
	PF	0.526 ± 0.327	0.064 ± 0.004	**2.066 ± 0.177** **b#**	**27.908 ± 4.634** **b#**	<0.001	0.023 ± 0.009

The concentration factors (CF) were calculated for all studied metals in organisms from all stations, however in different periods of sampling. The CF values may be ranked by the following order: Zn>Cu>Fe>Mn>Cd>Pb (**Table 3.5**).

It was possible to obtain results for the copper concentration factor in all seasons, except in winter, and only in a few stations (**Table 3.5**). The highest CF values were found in summer at AR station (1381 $L.kg^{-1}$ ± 242, **Table 3.5**) and in the other seasons and stations Cu CF values were considerably lower, ranging between 4.4 L.kg^{-1} ± 4.1 and 228.5 L.kg^{-1} ± 97.7. The Two-Way ANOVA test revealed effects between seasons and stations in Cu concentration factor in the organisms (Bonferroni test: ρ-value – 0.000, **Attachment 8**), with significant differences at AR station between summer and the other three seasons (Bonferroni test results: ρ-values – 0.000, **Attachment 8**). In addition, significant differences were found in summer between AR and CM stations (Bonferroni test: ρ-value – 0.000, **Attachment 8**).

The concentration factors (CF) for iron were possible to calculate in the organisms from all sampling stations and seasons. The highest Fe CF values were found in animals from CM station in the winter (252 L.kg^{-1} ± 121) and spring (185 L.kg^{-1} ± 10) while the lowest values were obtained in the autumn at AR station (6 L.kg^{-1} ± 2) and in the winter at BB station (7 L.kg^{-1} ± 1) (**Table 3.5**). The Two-Way ANOVA revealed effects between seasons and stations in Fe concentration factor in the organisms (ρ-value – 0.000, **Attachment 8**). The winter was characterized by the highest Fe CF values in animals from CM station, which were significantly different from the values obtained in this season at BB and AR stations (Bonferroni test results: ρ-values – 0.000, **Attachment 8**). Also, statistically significant differences were observed at CM station between summer and winter and between summer and spring (Bonferroni test results: ρ-values – 0.001 and 0.037, respectively, **Attachment 8**). In addition, the results for autumn at CM station were also significantly different from the values obtained in winter and spring (Bonferroni test results: ρ-values – 0.000 and 0.012, respectively, **Attachment 8**). As mentioned before, in the spring, the CF values were relatively high in the organisms from BB and CM stations and the post-hoc test results confirmed this showing significant differences between CM and PF stations (Bonferroni test: ρ-value – 0.011, **Attachment 8**). Also, significant differences were found at BB station between spring and winter (Bonferroni test: ρ-value – 0.047, **Attachment 8**).

The manganese (Mn) concentration factor (CF) was possible to calculate in *H. diversicolor* specimens in all seasons, except for summer, and in all stations however in different moments. The highest Mn CF values were found in the spring at AR (260 L.kg^{-1} ± 19) and CM (157 L.kg^{-1} ± 18) stations (**Table 3.5**). The Two-Way ANOVA revealed effects between seasons and stations in the Mn concentration factor from the organisms (ρ-value – 0.000, **Attachment 8**). In fact, significant differences were found in spring between AR and the other three stations (Bonferroni test results: ρ-values – 0.000), also between CM and the other three stations (Bonferroni test results: ρ-values – 0.000). Additionally, the post-hoc test results showed significant differences at AR and

CM stations between the spring and the other three seasons (Bonferroni test: ρ-values – 0.000, **Attachment 8**). Also, at BB station significant differences were found between spring and autumn and spring and winter (Bonferroni test: ρ-values – 0.000, **Attachment 8**). At PF station the highest CF values were found in spring and the post-hoc test showed significant differences between autumn and spring (Bonferroni test: ρ-value – 0.000, **Attachment 8**).

The Zn concentration factors were only obtained in winter and spring and the highest values were found at CM station in spring (14177 $L.kg^{-1}$ ± 766) followed by AR station in winter (4249 $L.kg^{-1}$ ± 766). The Two-Way ANOVA revealed effects between seasons and stations in Zn concentration factor of the organisms (ρ-value – 0.000, **Attachment 8**). The post-hoc test showed significant differences at AR station between winter and the other three seasons (Bonferroni test: ρ-values – 0.000, **Attachment 8**). Also, at CM station significant differences were found between spring and the other three seasons (Bonferroni test: ρ-values – 0.000, **Attachment 8**). At PF station it was only possible to obtain values in the spring and significant differences were found between the spring and autumn (Bonferroni test: ρ-value – 0.000, **Attachment 8**). As mentioned before, the highest values were found at AR station in the winter and the post-hoc test supported this showing significant differences in the winter between AR station and the stations BB and CM (ρ-values – 0.000, **Attachment 8**). In the spring significant differences were found between PF and the other three stations (Bonferroni test: ρ-values between 0.000 and 0.001, **Attachment 8**) and between CM and the other three stations (Bonferroni test: ρ-values – 0.000, **Attachment 8**).

The concentration factors (CF) of *H. diversicolor* for cadmium (Cd) were possible to calculate in all seasons and sampling stations, except for CM and PF stations in the spring. The highest Cd CF values were found in autumn (30 $L.\mu g^{-1}$ ± 8) and winter (13 $L.\mu g^{-1}$ ± 5) in the animals from AR station, while in the other seasons and stations Cd CF values were lower and similar (**Table 3.5**). The Two-Way ANOVA test revealed effects between seasons and stations (ρ-value – 0.000, **Attachment 8**). The post-hoc test results confirmed this showing significant differences at AR station between the autumn and the other three seasons (Bonferroni test results: ρ-values between 0.000 and 0.001, **Attachment 8**). Also, at AR station, significant differences were found between winter and spring (Bonferroni test: ρ-value – 0.040, **Attachment 8**). In all seasons, at BB, CM and PF stations the CF values were lower comparing with AR station and the post-hoc test results showed significant differences in the autumn between AR station and the other three stations (Bonferroni test: ρ-values – 0.000, **Attachment 8**). The same behavior was observed in the winter and significant differences were found between AR and BB stations (Bonferroni test: ρ-value – 0.015, **Attachment 8**) and between AR and CM stations (Bonferroni test: ρ-value – 0.016, **Attachment 8**).

The lead concentration factors (CF) were only obtained in autumn and winter and the highest Pb CF values were found in the autumn at AR station (2 $L.\mu g^{-1}$ ± 0.5), followed by BB station (0.9 $L.\mu g^{-1}$ ± 0.5), while the lowest value was found at CM station also in the autumn (0.07 $L.\mu g^{-1}$ ± 0.06) (**Table 3.5**). The Two-Way ANOVA test revealed effects between seasons and stations (ρ-value – 0.000, **Attachment 8**). To support the results the post-hoc test showed significant differences in the autumn between BB and the other three stations and between AR and the other stations (Bonferroni test results: BB and AR stations ρ-value – 0.036; BB and CM stations ρ-value – 0.039; BB and PF stations ρ-value – 0.021; AR and CM stations ρ-value – 0.000; and AR and PF stations ρ-value – 0.000, **Attachment 8**). Additionally, significant differences were found between autumn and spring at BB station (Bonferroni test: ρ-value – 0.010, **Attachment 8**) and at AR station significant differences were found between autumn and the other three seasons (Bonferroni test: ρ-values – 0.000, **Attachment 8**).

Table 3.5 – Concentration factors (CF) of Copper (Cu), Iron (Fe), Manganese (Mn), Zinc (Zn), Cadmium (Cd) and Lead (Pb) of *Hediste diversicolor* collected at Óbidos Lagoon during the study period. All values were expressed as mean ± standard deviation (N=7 in the summer at AR and CM stations; N=10, in the autumn and spring at all stations and in the winter at BB, AR and CM stations). Sampling sites: *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF). Limits of detection in micrograms per litre: 0.4 for Cu, 0.035 for Mn; 0.1 for Zn; 0.001 for Cd; and 0.06 for Pb. The symbols (*, # and +) indicate significant differences between seasons in the different stations and the letters (a, b and c) indicate significant differences between stations in each season. The symbols and letters combination correspond to ρ-value<0.050 (Bonferroni test); absence indicates that no significant differences were observed.

Seasons/ Stations		**Essentials metals**				**Nonessential metals**	
		CF_Cu (L.kg^{-1})	**CF_Fe (L.kg^{-1})**	**CF_Mn (L.kg^{-1})**	**CF_Zn (L.kg^{-1})**	**CF_Cd (L.μg^{-1})**	**CF_Pb (L.μg^{-1})**
Summer	**BB**	–	–	–	–	–	–
	AR	1381 ± 241.6 **a***	68.1 ± 7.5	<0.035	<0.1	6.5 ± 3.3 ***+**	<0.06
	CM	4.4 ± 4.1 **b**	24.4 ± 2.1 *****	<0.035	<0.1	5.8 ± 2.6	<0.06
	PF	–	–	–	–	–	–
Autumn	**BB**	73.2 ± 46.1	20.5 ± 2.2 *****	<0.035	<0.1	3.9 ± 2.7 **a**	0.9 ± 0.5 **a**
	AR	228.5 ± 97.8 #	6.4 ± 1.7	<0.035	<0.1	30.1 ± 7.9 **b#**	1.7 ± 0.5 **b***
	CM	9.8 ± 3.9	19.95 ± 1.7 *****	2.4 ± 2.2 *****	<0.1	0.2 ± 0.1 **a**	0.07 ± 0.06 **c**
	PF	<0.4	50.7 ± 2.4	9.6 ± 8.3 *****	<0.1	1.5 ± 0.3 **a**	<0.06
Winter	**BB**	<0.4	6.8 ± 1.0 **a***	<0.035	300.3 ± 96.6 **a**	0.4 ± 0.2 **a**	0.3 ± 0.2
	AR	<0.4	9.8 ± 2.4 **a**	<0.035	4248.7 ± 766.2 **b***	12.7 ± 4.9 **b+**	0.3 ± 0.1 #
	CM	<0.4	251.5 ± 120.6 **b#**	15.2 ± 12.5 *****	<0.1	0.450 ± 0.460 **a**	0.2 ± 0.1
	PF	–	–	–	–	–	–
Spring	**BB**	<0.4	134.7 ± 18.5 **ab#**	74.6 ± 13.9 **a**	<0.1	0.7 ± 0.5	<0.06
	AR	<0.4	80.8 ± 6.4 **ab**	260.3 ± 18.9 **b**	1398.5 ± 403.7 **a#**	0.8 ± 0.5 *****	<0.06
	CM	<0.4	184.96 ± 10.42 **a#**	157.2 ± 17.6 **c#**	14176 ± 766 **b**	<0.001	<0.06
	PF	136.7 ± 85.0	18.5 ± 1.0 **b**	78.2 ± 6.7 **a#**	3636.0 ± 603.8 **c**	<0.001	<0.06

3.4 The influence of the presence of trace metals in biological parameters

The effect of the presence of the distinct trace metals on *H. diversicolor* and on the environmental samples was evaluated through the Spearman's correlation technique ($\rho_{spearman}$) and 42 significant (ρ-value < 0.05 and ρ-spearman > 0.5) relationships were found (**Tables 3.6-3.10**).

Considering the biometrical parameters, modest and positive correlations were found between the L3 measurements and body size (ρ-spearman = 0.513, ρ-value < 0.05), and the L3 and the wet weights (ρ-spearman = 0.528, ρ-value < 0.05). As expected, the spearman correlation technique showed a strong positive correlation between body size and wet weights (ρ-spearman = 0.836, ρ-value < 0.05). Additionally, a modest and positive correlation was found between L3 and $[Mn]_{Org}$ (ρ-spearman = 0.665, ρ-value < 0.05); while a modest and negative correlation was observed between $[Pb]_{Org}$ and wet weights (ρ-spearman = - 0.606, ρ-value < 0.05) (**Table 3.6**).

It was possible to find different correlations between metal concentrations in the different environmental and biological samples. The $[Cu]_{Sed}$ showed a very strong positive correlation with $[Fe]_{Sed}$ (ρ-spearman = 0.933, ρ-value < 0.05), also strong positive correlations with $[Mn]_{Sed}$ (ρ-spearman = 0.844, ρ-value < 0.05) and with $[Zn]_{Sed}$ (ρ-spearman = 0.858, ρ-value < 0.05). Additionally, $[Fe]_{Sed}$ showed a very strong positive correlation with $[Mn]_{Sed}$ (ρ-spearman = 0.914, ρ-value < 0.05) and a strong positive correlation with $[Zn]_{Sed}$ (ρ-spearman = 0.885, ρ-value < 0.05). Also, $[Mn]_{Sed}$ and $[Zn]_{Sed}$ showed a strong positive correlation (0.861, ρ-value < 0.05) (**Table 3.6**). Considering the concentrations of metals suspended in the water samples, $[Cu]_{Sus}$ showed modest positive correlations with $[Fe]_{Sus}$ (ρ-spearman – 0.623, ρ-value < 0.05) and $[Mn]_{Sus}$ (ρ-spearman = 0.541, ρ-value < 0.05) (**Table 3.7**). Additionally, $[Fe]_{Sus}$ showed a strong positive correlation with $[Mn]_{Sus}$ (ρ-spearman = 0.831, ρ-value < 0.05), while $[Fe]_{Dis}$ and $[Pb]_{Sus}$ showed a positive modest correlation (ρ-spearman = 0.637, ρ-value < 0.05) (**Table 3.8**). As for the relationships between biological and environmental samples, a modest positive correlation was observed between the $[Cd]_{Org}$ and $[Pb]_{Org}$ (ρ-spearman = 0.636, ρ-value < 0.05) and as expected, $[Pb]_{Sus}$ and $[Pb]_{Org}$ showed a significant positive correlation (ρ-spearman = 0.517, ρ-value < 0.05) (**Table 3.7**). Modest positive correlations were also found between $[Pb]_{Sus}$ and Pb_{CF} (ρ-spearman = 0.551, ρ-value < 0.05), and between $[Pb]_{Sus}$ and Pb_{BAF} (ρ-spearman = 0.573, ρ-value < 0.05) (**Table 3.8**).

Regarding the influence of biological and environmental variables on the bioaccumulation and concentration factors of *H. diversicolor*, modest positive correlations were observed between Mn_{BAF} and body size (ρ-spearman = 0.603, ρ-value < 0.05) and Cu_{BAF} and wet weight (ρ-spearman = 0.601, ρ-value < 0.05). As expected, the metal concentration in the organism and the bioaccumulation factor for the respective metal were correlated, then very strong positive correlations were found between Cu_{BAF} and $[Cu]_{Org}$ (ρ-spearman = 0.977, ρ-value < 0.05); between Mn_{BAF} and $[Mn]_{Org}$ (ρ-spearman = 0.948, ρ-value < 0.05); and between Pb_{BAF} and $[Pb]_{Org}$ (ρ-spearman = 0.951, ρ-value < 0.05). Similar results were found for concentrations factors, namely between Cd_{CF} and $[Cd]_{Org}$ (ρ-spearman = 0.942, ρ-value < 0.05); between Mn_{CF} and $[Mn]_{Org}$ (ρ-spearman = 0.985, ρ-value < 0.05); and between Cd_{CF} and $[Cd]_{Org}$ (ρ-spearman = 0.942, ρ-value < 0.05) (**Table 3.9**). Also, modest positive correlations were found between Cu_{CF} and $[Cu]_{Org}$ (ρ-spearman = 0.650, ρ-value < 0.05) and between Pb_{CF} and $[Pb]_{Org}$ (ρ-spearman = 0.596, ρ-value < 0.05) (**Table 3.9**). In addition, a strong positive correlation was found between Mn_{CF} and Mn_{BAF} (ρ-spearman = 0.949, ρ-value < 0.05) (**Table 3.9)**. Also, modest positive correlations were found between Cu_{CF} and Cu_{BAF} (ρ-spearman = 0.635, ρ-value < 0.05) and between Pb_{CF} and Pb_{BAF} (ρ-spearman = 0.576, ρ-value < 0.05) (**Table 3.9**).

Correlations were also found between different metals' bioacumulation and concentration factors such as modest positive correlations between Fe_{CF} and Mn_{CF} (ρ-spearman = 0.578, ρ-value < 0.05), Zn_{CF} and Mn_{BAF} (ρ-spearman = 0.523, ρ-value < 0.05), and Cd_{CF} and Pb_{BAF} (ρ-spearman = 0.554, ρ-value < 0.05) (**Table 3.9**). In addition, negative modest correlations were also found between Fe_{CF} and Pb_{CF} (ρ-spearman = -0.562, ρ-value < 0.05); Mn_{CF} and Pb_{CF} (ρ-spearman = -0.551, ρ-value < 0.05); and between Pb_{CF} and Mn_{BAF} (ρ-spearman = -0.551, ρ-value < 0.05) (**Table 3.9**). Finally, a modest negative correlation was found between Zn_{BAF} and Fe_{BAF} (ρ-spearman = - 0.631, ρ-value < 0.05) (**Table 3.9**).

Additionally, correlations were found between metal concentrations and different bioaccumulation and concentrations factors. Modest positive correlations were found between Fe_{CF} and $[Mn]_{Org}$ (ρ-spearman = 0.524, ρ-value < 0.05); Cd_{CF} and $[Pb]_{Org}$ (ρ-spearman = 0.567, ρ-value < 0.05); and Pb_{BAF} and $[Cd]_{Org}$ (ρ-spearman = 0.584, ρ-value > 0.05) (**Table 3.9**). Also, a modest negative correlation was found between Pb_{CF} and $[Mn]_{Org}$ (ρ-spearman = -0.551, ρ-value < 0.05) (**Table 3.9**).

Furthermore, spearman test showed correlations between concentration and bioaccumulation factors and the *H. diversicolor* biometrical parameters. Modest positive correlations were found between Mn_{BAF} and L3 length (ρ-spearman = 0.702, ρ-value < 0.05) and between Mn_{CF} and L3 length (ρ-spearman = 0.679, ρ-value < 0.05) (**Table 3.10**). In addition, a modest negative correlation was found between Pb_{BAF} and wet weight (ρ-spearman = -0.517, ρ-value < 0.05) (**Table 3.10**) and the only correlation found for lipid content was a modest negative correlation with $[Pb]_{Sed}$ (ρ-spearman = -0.555, ρ-value > 0.05) (**Table 3.11**).

Table 3.6 – Spearman correlation matrix ($\rho_{spearman}$) for different variables of *H. diversicolor* and environmental samples, such as metal concentration in the organisms, represented as $[Cu]_{Org}$, $[Fe]_{Org}$, $[Mn]_{Org}$, $[Zn]_{Org}$, $[Cd]_{Org}$, $[Pb]_{Org}$ and the biometrical parameters (L3), body size (BS), wet weight (WW); the metal concentrations in the sediment $[Cu]_{Sed}$, $[Fe]_{Sed}$, $[Mn]_{Sed}$, $[Zn]_{Sed}$, $[Cd]_{Sed}$, and $[Pb]_{Sed}$ and dissolved in the water column $[Fe]_{Dis}$, $[Mn]_{Dis}$ and $[Cd]_{Dis}$. Significant correlations (ρ-value < 0.05) are represented in bold. (N=124 for metals in *H.diversicolor*; N=123 for biometrical parameters; N=48 for metals in the sediment and N=46 for dissolved metals).

SPEARMAN																		
	$[Cu]_{Org}$	**$[Fe]_{Org}$**	**$[Mn]_{Org}$**	**$[Zn]_{Org}$**	**$[Cd]_{Org}$**	**$[Pb]_{Org}$**	**L3**	**BS**	**WW**	**$[Cu]_{Sed}$**	**$[Fe]_{Sed}$**	**$[Mn]_{Sed}$**	**$[Zn]_{Sed}$**	**$[Cd]_{Sed}$**	**$[Pb]_{Sed}$**	**$[Fe]_{Dis}$**	**$[Mn]_{Dis}$**	**$[Cd]_{Dis}$**
$[Cu]_{Org}$	1																	
$[Fe]_{Org}$	0.009	1																
$[Mn]_{Org}$	-0.116	**0.221**	1															
$[Zn]_{Org}$	**0.405**	0.122	-0.049	1														
$[Cd]_{Org}$	-0.069	-0.130	**-0.270**	-0.166	1													
$[Pb]_{Org}$	**-0.218**	-0.057	**-0.242**	**-0.263**	**0.636**	1												
L3	-0.068	0.134	**0.665**	-0.037	**-0.431**	**-0.438**	1											
BS	0.087	-0.070	**0.365**	0.001	**-0.254**	**-0.462**	**0.513**	1										
WW	**0.238**	-0.084	**0.222**	0.000	**-0.375**	**-0.606**	**0.528**	**0.836**	1									
$[Cu]_{Sed}$	-0.057	-0.100	-0.058	-0.136	0.067	-0.054	0.132	0.040	-0.002	1								
$[Fe]_{Sed}$	-0.104	-0.125	-0.010	-0.102	-0.012	-0.083	0.044	-0.007	-0.053	**0.933**	1							
$[Mn]_{Sed}$	-0.059	-0.097	-0.096	-0.056	0.003	-0.002	0.084	-0.068	-0.053	**0.844**	**0.914**	1						
$[Zn]_{Sed}$	-0.015	-0.046	-0.084	-0.102	0.058	-0.008	0.118	0.006	0.001	**0.858**	**0.885**	**0.861**	1					
$[Cd]_{Sed}$	-0.091	0.141	**0.323**	-0.159	-0.167	-0.139	0.029	-0.082	-0.043	0.046	0.074	0.015	0.116	1				
$[Pb]_{Sed}$	0.255	**-0.395**	-0.088	-0.171	**0.303**	0.214	-0.132	-0.223	-0.014	0.181	0.220	0.241	0.136	0.046	1			
$[Fe]_{Dis}$	0.211	-0.250	-0.082	-0.123	**0.311**	**0.367**	0.231	0.125	0.126	**0.367**	**0.394**	**0.342**	**0.373**	-0.061	0.252	1		
$[Mn]_{Dis}$	0.269	0.169	-0.138	0.119	0.200	0.141	0.038	-0.239	-0.129	**0.324**	**0.313**	**0.469**	**0.414**	0.147	**0.392**	0.167	1	
$[Cd]_{Dis}$	-0.088	0.024	0.150	-0.136	0.067	0.267	**0.421**	0.004	0.047	0.232	0.235	0.203	0.255	**0.298**	0.048	0.238	0.087	1

Table 3.7 – Spearman correlation matrix ($\rho_{spearman}$) for different variables of *H. diversicolor* and environmental samples, such as the metal concentration in the organisms, represented as $[Cu]_{Org}$, $[Fe]_{Org}$, $[Mn]_{Org}$, $[Zn]_{Org}$, $[Cd]_{Org}$ and $[Pb]_{Org}$, and the biometrical parameters (L3), body size (BS), wet weight (WW); and the metal concentrations found suspended in the water column $[Cu]_{Sus}$, $[Fe]_{Sus}$, $[Mn]_{Sus}$, $[Zn]_{Sed}$, $[Cd]_{Sus}$, and $[Pb]_{Sus}$. Significant correlations (ρ-value < 0.05) are represented in bold. (N=124 for metals in *H.diversicolor*; N=123 for biometrical parameters; N=48 for metals in the sediment and N=46 for dissolved metals.

SPEARMAN															
	$[Cu]_{Org}$	**$[Fe]_{Org}$**	**$[Mn]_{Org}$**	**$[Zn]_{Org}$**	**$[Cd]_{Org}$**	**$[Pb]_{Org}$**	**L3**	**BS**	**WW**	**$[Cu]_{Sus}$**	**$[Fe]_{Sus}$**	**$[Mn]_{Sus}$**	**$[Zn]_{Sus}$**	**$[Cd]_{Sus}$**	**$[Pb]_{Sus}$**
$[Cu]_{Org}$	1														
$[Fe]_{Org}$	0.009	1													
$[Mn]_{Org}$	-0.116	**0.221**	1												
$[Zn]_{Org}$	**0.405**	0.122	-0.049	1											
$[Cd]_{Org}$	-0.069	-0.130	**-0.270**	-0.166	1										
$[Pb]_{Org}$	**-0.218**	-0.057	**-0.242**	**-0.263**	**0.636**	1									
L3	-0.068	0.134	**0.665**	-0.037	**-0.431**	**-0.438**	1								
BS	0.087	-0.070	**0.365**	0.001	**-0.254**	**-0.462**	**0.513**	1							
WW	**0.238**	-0.084	**0.222**	0.000	**-0.375**	**-0.606**	**0.528**	**0.836**	1						
$[Cu]_{Sus}$	-0.080	0.223	-0.006	0.161	0.225	0.082	-0.023	-0.069	-0.060	1					
$[Fe]_{Sus}$	0.138	0.255	-0.073	0.102	0.244	0.076	0.071	0.014	-0.056	**0.623**	1				
$[Mn]_{Sus}$	0.054	0.259	0.038	0.100	0.189	0.076	0.000	-0.220	-0.221	**0.541**	**0.831**	1			
$[Zn]_{Sus}$	-0.027	-0.086	0.100	0.066	-0.004	-0.169	-0.016	**0.381**	**0.434**	0.161	0.284	0.202	1		
$[Cd]_{Sus}$	0.267	0.243	-0.092	0.284	-0.063	-0.166	-0.135	0.098	0.026	-0.006	0.144	0.130	0.258	1	
$[Pb]_{Sus}$	0.078	-0.141	-0.209	-0.141	**0.455**	**0.517**	0.249	-0.030	-0.082	**0.320**	**0.479**	**0.449**	0.211	0.023	1

Table 3.8 – Spearman correlation matrix ($\rho_{spearman}$) for different variables of *H. diversicolor* and environmental samples, such as the bioaccumulation and concentration factors, represented as Cu$_{BAF}$, Fe$_{BAF}$, Mn$_{BAF}$, Zn$_{BAF}$, Cd$_{BAF}$, Pb$_{BAF}$, Fe$_{CF}$, Mn$_{CF}$ and Cd$_{CF}$; the metal concentrations found suspended,[Cu]$_{Sus}$, [Fe]$_{Sus}$, [Mn]$_{Sus}$, [Zn]$_{Sed}$, [Cd]$_{Sus}$, and [Pb]$_{Sus,}$ and dissolved in the water column, [Fe]$_{Dis}$, [Mn]$_{Dis}$ and [Cd]$_{Dis}$. Significant correlations (ρ-value < 0.05) are represented in bold. (N=124 for BAF and CF; N=46 for suspended and dissolved metals in the water column).

SPEARMAN

	Cu$_{BAF}$	**Fe$_{BAF}$**	**Mn$_{BAF}$**	**Zn$_{BAF}$**	**Cd$_{BAF}$**	**Pb$_{BAF}$**	**Cu$_{CF}$**	**Fe$_{CF}$**	**Mn$_{CF}$**	**Zn$_{CF}$**	**Cd$_{CF}$**	**Pb$_{CF}$**	**[Cu]$_{Sus}$**	**[Fe]$_{Sus}$**	**[Mn]$_{Sus}$**	**[Zn]$_{Sus}$**	**[Cd]$_{Sus}$**	**[Pb]$_{Sus}$**	**[Fe]$_{Dis}$**	**[Mn]$_{Dis}$**	**[Cd]$_{Dis}$**
Cu$_{BAF}$	1																				
Fe$_{BAF}$	-0.062	1																			
Mn$_{BAF}$	-0.01	0.044	1																		
Zn$_{BAF}$	0.153	**-0.631**	-0.194	1																	
Cd$_{BAF}$	-0.165	**0.397**	-0.186	-0.221	1																
Pb$_{BAF}$	-0.156	**-0.370**	**-0.368**	**0.329**	0.208	1															
Cu$_{CF}$	**0.635**	0.137	-0.151	0.164	-0.171	0.069	1														
Fe$_{CF}$	0.130	**0.336**	**0.488**	**-0.285**	**-0.201**	**-0.255**	-0.140	1													
Mn$_{CF}$	-0.051	0.132	**0.949**	0.027	-0.165	**-0.241**	**-0.216**	**0.578**	1												
Zn$_{CF}$	-0.081	**0.192**	**0.523**	**0.359**	-0.074	**-0.180**	**-0.191**	0.035	**0.480**	1											
Cd$_{CF}$	0.062	**-0.224**	**-0.451**	**0.191**	**0.236**	**0.554**	0.110	**-0.280**	**-0.379**	**-0.195**	1										
Pb$_{CF}$	**-0.178**	**-0.355**	**-0.551**	0.100	0.083	**0.576**	-0.014	**-0.562**	**-0.551**	-0.094	**0.383**	1									
[Cu]$_{Sus}$	-0.058	-0.161	-0.013	0.002	0.288	0.056	-0.081	0.097	-0.006	-	0.202	0.012	1								
[Fe]$_{Sus}$	0.155	-0.273	-0.083	0.275	0.060	0.065	0.089	0.171	-0.073	-	0.243	-0.022	**0.623**	1							
[Mn]$_{Sus}$	0.088	-0.168	0.036	0.203	0.091	0.038	0.114	0.224	0.038	-	0.184	-0.060	**0.541**	**0.831**	1						
[Zn]$_{Sus}$	-0.005	0.004	0.091	0.024	-0.151	-0.128	-0.086	-0.089	0.100	-	0.017	0.065	0.161	0.284	0.202	1					
[Cd]$_{Sus}$	0.261	-0.055	-0.092	0.124	-0.107	-0.197	0.291*	0.205	-0.092	-	-0.068	-0.126	-0.006	0.144	0.130	0.258	1				
[Pb]$_{Sus}$	0.092	**-0.507**	-0.209	**0.432**	-0.107	**0.573**	0.052	**-0.469**	-0.203	-	**0.458**	**0.551**	**0.320**	**0.479**	**0.449**	0.211	0.023	1			
[Fe]$_{Dis}$	-0.255	-0.087	0.286	-0.146	**0.419**	0.185	0.159	**-0.293**	-0.082	-	**0.338**	**0.441**	0.153	**0.381**	**0.333**	**0.326**	-0.079	**0.637**	1		
[Mn]$_{Dis}$	0.133	-0.138	**0.346**	-0.007	0.026	**0.317**	**0.364**	**0.452**	-0.138	-	0.193	-0.170	-0.107	0.144	**0.392**	-0.010	0.171	0.066	0.167	1	
[Cd]$_{Dis}$	0.011	0.163	0.068	0.047	**0.331**	-0.056	-0.003	-0.207	0.150	-	0.081	**0.389**	-0.094	-0.148	-0.054	0.024	0.112	0.199	0.238	0.087	1

Table 3.9 – Spearman correlation matrix ($\rho_{spearman}$) for different variables of *H. diversicolor* and environmental samples, such as the metal concentration in the organism, represented as $[Cu]_{Org}$, $[Fe]_{Org}$, $[Mn]_{Org}$, $[Zn]_{Org}$, $[Cd]_{Org}$ and $[Pb]_{Org}$; the bioaccumulation and concentration factors, represented as Cu_{BAF}, Fe_{BAF}, Mn_{BAF}, Zn_{BAF}, Cd_{BAF}, Pb_{BAF}, Fe_{CF}, Mn_{CF}, Cd_{CF}; and the biometrical parameters (L3), body size (BS), wet weight (WW); Significant correlations (ρ-value < 0.05) are represented in bold. (N=124 for BAF and CF, and metals in *H. diversicolor*; and N=123 for biometrical parameters).

SPEARMAN																					
	$[Cu]_{Org}$	**$[Fe]_{Org}$**	**$[Mn]_{Org}$**	**$[Zn]_{Org}$**	**$[Cd]_{Org}$**	**$[Pb]_{Org}$**	**Cu_{BAF}**	**Fe_{BAF}**	**Mn_{BAF}**	**Zn_{BAF}**	**Cd_{BAF}**	**Pb_{BAF}**	**Cu_{CF}**	**Fe_{CF}**	**Mn_{CF}**	**Zn_{CF}**	**Cd_{CF}**	**Pb_{CF}**	**L3**	**BS**	**WW**
$[Cu]_{Org}$	1																				
$[Fe]_{Org}$	0.009	1																			
$[Mn]_{Org}$	-0.116	**0.221**	1																		
$[Zn]_{Org}$	**0.405**	0.122	-0.049	1																	
$[Cd]_{Org}$	-0.069	-0.130	**-0.270**	-0.166	1																
$[Pb]_{Org}$	**-0.218**	-0.057	**-0.242**	**-0.263**	**0.636**	1															
Cu_{BAF}	**0.977**	-0.008	-0.082	**0.388**	-0.077	**-0.248**	1														
Fe_{BAF}	0.040	**0.328**	0.110	-0.016	**-0.271**	**-0.294**	-0.062	1													
Mn_{BAF}	-0.044	**0.212**	**0.948**	-0.036	**-0.366**	**-0.369**	-0.010	0.044	1												
Zn_{BAF}	**0.178**	0.019	0.093	**0.484**	0.106	0.127	0.153	**-0.631**	-0.194	1											
Cd_{BAF}	**-0.274**	-0.010	-0.156	**-0.230**	**0.405**	**0.280**	-0.165	**0.397**	-0.186	-0.221	1										
Pb_{BAF}	-0.148	-0.018	**-0.224**	**-0.250**	**0.584**	**0.951**	-0.156	**-0.370**	**-0.368**	**0.329**	0.208	1									
Cu_{CF}	**0.650**	0.067	**-0.187**	**0.201**	-0.001	0.016	**0.635**	0.137	-0.151	0.164	-0.171	0.069	1								
Fe_{CF}	0.117	**0.474**	**0.524**	0.094	**-0.271**	**-0.288**	0.130	**0.336**	**0.488**	**-0.285**	**-0.201**	**-0.255**	-0.140	1							
Mn_{CF}	-0.093	**0.208**	**0.985**	-0.049	**-0.268**	**-0.253**	-0.051	0.132	**0.949**	0.027	-0.165	**-0.241**	**-0.216**	**0.578**	1						
Zn_{CF}	-0.146	0.091	**0.452**	0.139	-0.089	-0.064	-0.081	**0.192**	**0.523**	**0.359**	-0.074	**-0.180**	**-0.191**	0.035	**0.480**	1					
Cd_{CF}	0.075	-0.173	**-0.380**	-0.101	**0.942**	**0.567**	0.062	**-0.224**	**-0.451**	**0.191**	**0.236**	**0.554**	0.110	**-0.280**	**-0.379**	**-0.195**	1				
Pb_{CF}	-0.169	**-0.210**	**-0.551**	-0.085	**0.365**	**0.596**	**-0.178**	**-0.355**	**-0.551**	0.100	0.083	**0.576**	-0.014	**-0.562**	**-0.551**	-0.094	**0.383**	1			
L3	-0.068	0.134	**0.665**	-0.037	**-0.431**	**-0.438**	-0.115	-0.033	**0.386**	**0.246**	0.038	0.173	-0,152	**0,438**	**0,669**	**0,395**	**-0,474**	**-0,446**	1		
BS	0.087	-0.070	**0.365**	0.001	**-0.254**	**-0.462**	-0.127	0.002	**0.603**	0.055	-0.020	-0.133	**-0,231**	**0,321**	**0,391**	**0,334**	**-0,242**	**-0,236**	**0.513**	1	
WW	**0.238**	-0.084	**0.222**	0.000	**-0.375**	**-0.606**	-0.067	0.068	**0.601**	-0.021	-0.196	-0.088	-0,030	**0,341**	**0,254**	0,074	**-0,275**	**-0,328**	**0.528**	**0.836**	1

Table 3.10 – Spearman correlation matrix ($\rho_{spearman}$) for different variables of *H. diversicolor* and the environmental samples, such as the bioaccumulation and concentration factors, represented as Cu_{BAF}, Fe_{BAF}, Mn_{BAF}, Zn_{BAF}, Cd_{BAF}, Pb_{BAF}, Fe_{CF}, Mn_{CF} and Cd_{CF}; and the metal concentrations in the sediment, represented as $[Cu]_{Sed}$, $[Fe]_{Sed}$, $[Mn]_{Sed}$, $[Zn]_{Sed}$, $[Cd]_{Sed}$, and $[Pb]_{Sed}$. Significant correlations (ρ-value < 0.05) are represented in bold (N=124 for BAF and CF; N=123 for biometrical parameters; and N=48 for metals in the sediment).

SPEARMAN																					
	Cu$_{BAF}$	**Fe$_{BAF}$**	**Mn$_{BAF}$**	**Zn$_{BAF}$**	**Cd$_{BAF}$**	**Pb$_{BAF}$**	**Cu$_{CF}$**	**Fe$_{CF}$**	**Mn$_{CF}$**	**Zn$_{CF}$**	**Cd$_{CF}$**	**Pb$_{CF}$**	**[Cu]$_{Sed}$**	**[Fe]$_{Sed}$**	**[Mn]$_{Sed}$**	**[Zn]$_{Sed}$**	**[Cd]$_{Sed}$**	**[Pb]$_{Sed}$**	**L3**	**BS**	**WW**
Cu$_{BAF}$	1																				
Fe$_{BAF}$	-0.062	1																			
Mn$_{BAF}$	-0.010	0.044	1																		
Zn$_{BAF}$	0.153	**-0.631**	-0.194	1																	
Cd$_{BAF}$	-0.165	**0.397**	-0.186	-0.221	1																
Pb$_{BAF}$	-0.156	**-0.370**	**-0.368**	**0.329**	0.208	1															
Cu$_{CF}$	**0.635**	0.137	-0.151	0.164	-0.171	0.069	1														
Fe$_{CF}$	0.130	**0.336**	**0.488**	**-0.285**	**-0.201**	**-0.255**	-0.140	1													
Mn$_{CF}$	-0.051	0.132	**0.949**	0.027	-0.165	**-0.241**	**-0.216**	**0.578**	1												
Zn$_{CF}$	-0.081	**0.192**	**0.523**	**0.359**	-0.074	**-0.180**	**-0.191**	0.035	**0.480**	1											
Cd$_{CF}$	0.062	**-0.224**	**-0.451**	**0.191**	**0.236**	**0.554**	0.110	**-0.280**	**-0.379**	**-0.195**	1										
Pb$_{CF}$	**-0.178**	**-0.355**	**-0.551**	0.100	0.083	**0.576**	-0.014	**-0.562**	**-0.551**	-0.094	**0.383**	1									
[Cu]$_{Sed}$	-0.039	-0.032	-0.050	0.106	-0.046	-0.011	0.032	-0.147	-0.058	-	0.071	0.063	1								
[Fe]$_{Sed}$	-0.079	0.012	-0.001	0.107	-0.025	-0.070	-0.025	-0.122	-0.010	-	-0.016	0.001	**0.933**	1							
[Mn]$_{Sed}$	-0.001	0.010	-0.086	0.185	-0.025	-0.018	0.079	-0.126	-0.096	-	0.010	0.010	**0.844**	**0.914**	1						
[Zn]$_{Sed}$	0.036	0.030	-0.077	0.168	0.045	-0.026	0.095	-0.002	-0.084	-	0.048	-0.050	**0.858**	**0.885**	**0.861**	1					
[Cd]$_{Sed}$	-0.099	0.233	**0.321**	-0.011	-0.113	-0.175	-0.137	0.235	**0.323**	-	-0.177	-0.153	0.046	0.074	0.015	0.116	1				
[Pb]$_{Sed}$	0.276	0.090	-0.088	0.247	-0.210	0.173	0.266	-0.062	-0.088	-	**0.325**	0.119	0.181	0.220	0.241	0.136	0.046	1			
L3	0.003	**0.257**	**0.702**	-0.071	**-0.181**	**-0.397**	-0.152	**0.438**	**0.669**	**0.395**	**-0.474**	**-0.446**	0.132	0.044	0.084	0.118	0.029	-0.132	1		
BS	0.136	**0.207**	**0.478**	-0.107	**-0.177**	**-0.452**	**-0.231**	**0.321**	**0.391**	**0.334**	**-0.242**	**-0.236**	0.040	-0.007	-0.068	0.006	-0.082	-0.223	**0.513**	1	
WW	**0.290**	**0.339**	**0.384**	**-0.230**	**-0.225**	**-0.517**	-0.030	**0.341**	**0.254**	0.074	**-0.275**	**-0.328**	-0.002	-0.053	-0.053	0.001	-0.043	-0.014	**0.528**	**0.836**	1

Table 3.11 – Spearman correlation matrix ($\rho_{spearman}$) for different variables of *H. diversicolor* and environmental samples, such as the metal concentration in the organism, represented as $[Cu]_{Org}$, $[Fe]_{Org}$, $[Mn]_{Org}$, $[Zn]_{Org}$, $[Cd]_{Org}$ and $[Pb]_{Org}$; the bioaccumulation and concentration factors, represented as Cu_{BAF}, Fe_{BAF}, Mn_{BAF}, Zn_{BAF}, Cd_{BAF}, Pb_{BAF}, Fe_{CF}, Mn_{CF} and Cd_{CF}; the biometrical parameters (L3), body size (BS), wet weight (WW); and the lipid content (Lipids); the metal concentrations in the sediment, represented as $[Cu]_{Sed}$, $[Fe]_{Sed}$, $[Mn]_{Sed}$, $[Zn]_{Sed}$, $[Cd]_{Sed}$, $[Pb]_{Sed}$; and the metal concentrations found suspended, $[Cu]_{Sus}$, $[Fe]_{Sus}$, $[Mn]_{Sus}$, $[Zn]_{Sed}$, $[Cd]_{Sus}$, and $[Pb]_{Sus}$, and dissolved in the water column, $[Fe]_{Dis}$, $[Mn]_{Dis}$ and $[Cd]_{Dis}$. Significant correlations (ρ-value < 0.05) are represented in bold (N=124 for BAF and CF, and metals in *H. diversicolor*; N=56 for lipids; N=123 for biometrical parameters; N=48 for metals in the sediment; and N=46 for suspended and dissolved metals in the water column).

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	Lipids		**Lipids**
$[Cu]_{Org}$	-0.104	$[Cu]_{Sus}$	0.382
$[Fe]_{Org}$	-0.098	$[Fe]_{Sus}$	0.238
$[Mn]_{Org}$	0.164	$[Mn]_{Sus}$	0.313
$[Zn]_{Org}$	0.022	$[Zn]_{Sus}$	-0.070
$[Cd]_{Org}$	-0.150	$[Cd]_{Sus}$	-0.425
$[Pb]_{Org}$	-0.173	$[Pb]_{Sus}$	0.341
Cu_{BAF}	-0.106	$[Fe]_{Dis}$	0.374
Fe_{BAF}	0.104	$[Mn]_{Dis}$	-0.136
Mn_{BAF}	0.142	$[Cd]_{Dis}$	-0.215
Zn_{BAF}	-0.090	$[Cu]_{Sus}$	0.382
Cd_{BAF}	0.061	$[Fe]_{Sus}$	0.238
Pb_{BAF}	-0.046	$[Mn]_{Sus}$	0.313
Cu_{CF}	-0.173	$[Zn]_{Sus}$	-0.070
Fe_{CF}	-0.121	$[Cd]_{Sus}$	-0.425
Mn_{CF}	0.041	$[Pb]_{Sus}$	-0.555
Zn_{CF}	-0.176	$[Cu]_{Sed}$	-0.069
Cd_{CF}	-0.128	$[Fe]_{Sed}$	-0.074
Pb_{CF}	0.048	$[Mn]_{Sed}$	-0.158
L3	0.264	$[Zn]_{Sed}$	-0.184
BS	0.099	$[Cd]_{Sed}$	-0.148
WW	0.164	$[Pb]_{Sed}$	**-0.555**
Lipids	1	Lipids	1

4. Discussion

The environmental conditions at the sampling sites were characterized using the physicochemical parameters water temperature, salinity, pH and organic matter content analyses. Comparing water temperature, salinity and pH at BB (average: T = 19.1°C, S= 25.0 & pH= 7.5) and AR stations (average: T = 17.5°C, S= 20.5 & pH= 8.0) with those from CM (average: T = 19.9°C, S= 32.6 & pH= 8.1) and PF stations (average: T = 21.8°C, S= 31.7 & pH= 8.2), and considering the typical values of standard seawater (S = 35 & pH = 8), it was visible a higher influence of the tidal cycles at CM and PF stations. It is important to notice, that PF station is a small embayment on the left margin of the Lagoon and freshwater discharges are reduced when compared with the other stations. To the south, it is located the CM station, which is a confined arm and receives only a small freshwater discharge from the *Ameal* ditch. Between the two arms of the Óbidos Lagoon is located the AR station, which is the least restrained station, and where the rivers *Arnóia* and *Real* deliver their flows which are responsible for the highest freshwater discharge when compared to the other stations. In the right arm, is located the BB station which is another limited bay on the right margin, where the *Cal* and *Barrosa* rivers provide a substantial freshwater discharge (Malhadas *et al.*, 2014). These observations allow to conclude that PF and CM stations suffer a higher effect of the tidal cycles than AR and BB stations which easily may receive significant freshwater inputs. The Mann-Whitney test results corroborates this showing significant differences in salinity between AR station and the stations PF and CM. Also, Pereira *et al.* (2009c) and Carvalho et al. (2011) associated the lower salinity at BB station to freshwater inputs.

Being a coastal lagoon, this environment is more sensitive to the different freshwater discharges due to its semi-enclosed characteristics. The spatial and temporal fluctuations observed in the distinct physicochemical and biological properties in this type of ecosystems are ample (Crivelli *et al.*, 1995). The entry of freshwater on the lagoon increases with the precipitation which induces a decrease of salinity and water temperature. Also, the increase of nutrient inputs namely, organic matter, nitrogen and phosphorus to the lagoon via terrestrial runoffs increases the pH (Crivelli *et al.*, 1995).

In winter, all stations showed lower water temperatures and a tendency to lower salinities at BB and AR stations was noticed, which coincide with the high precipitation levels typical in this season. The Mann-Whitney test results support this observation, showing significant differences in water temperature between winter and the other seasons and significant differences in salinity between AR station and CM and PF stations. Also, lower water temperatures were found in the autumn, which also correspond to higher levels of precipitation. As expected, the higher water temperatures were observed in summer, and the statistical tests corroborate these results. The highest pH values were found in spring, followed by winter. The Mann-Whitney test results showed significant differences between spring and the seasons summer and autumn. The lack of significant differences between winter and the other seasons points to higher pH also in winter, which was expected due to the precipitation period. In addition, pH values ranged between 7.3 and 8.9 at all stations and seasons, except for BB in summer, which pH value was considerably lower (pH=5.3). As mentioned before, this station receives freshwater discharges from *Cal* river, which is receptor of domestic effluents from the rivers in *Caldas da Rainha* and *Gaeiras*. the higher domestic effluent discharges, characteristic of this period (Malhadas *et al*., 2014). These discharges from Cal river are probably responsible by this lower pH.

Benthic organisms are in direct contact with the sediment, where contaminants and organic matter accumulate (Chapman, 1990; Carvalho *et al*., 2006). Therefore, the quantification of the organic matter content in the sediments, on each station and season, was an imperative task. The highest levels of organic matter content in the sediment were found at BB (2.825% ± 0.4 on average) and PF stations (2.695% ± 0.6 on average) and the lowest at AR (0.890% ± 0.4 on average) and CM (0.936% ± 0.5 on average) stations. Previous studies report that the nutrient load and the longer residence times of water in the upper lagoon originated by weak tidal currents, reduces the dilution processes and enhances the accumulation of organic matter in sediments (Pereira *et al.*, 2009b; Pereira *et al.*, 2009c). Due to the embayment characteristic of BB and PF stations, favorable conditions for metal deposition are observed at these two stations, so it was expected that the organic matter content values were considerably higher in these stations. To support this, significant differences were found between the organic matter content from BB and the CM and AR stations, however in different seasons. Also, significant differences were found between the OMC from PF station and the AR and CM stations in different seasons.

As mentioned before, BB and AR stations receive freshwater from rivers which are associated to nutrient and contaminants discharges through the Óbidos Lagoon. At AR station, the *Arnóia* and *Real* rivers have a higher flow (3 $m^3.s^{-1}$) when compared with *Cal* river (0.08 $m^3.s^{-1}$), at CM station (Malhadas *et al.*, 2014). Pereira *et al.* (2010) considered BB station as eutrophic and the origin of highest OMC values at this station could be related with the enrichment by the old wastewater treatment (Malhadas *et al.*, 2014). Also, the variation in OMC between stations may be associated with the sediment composition. According to Carvalho *et al.* (2005), clay particles tend to bind higher quantities of organic matter and as a result, muddy areas are consistently richer in organic matter than sandy areas. To support this, the granulometry characterization performed in the present study revealed that CM and AR stations were constituted mostly by sand and BB and PF stations by silts. As expected, the highest OMC values were found at PF and BB stations, while at CM and AR stations OMC values were similar and lower. Also, at BB station the OMC values were similar in all seasons, however comparing with the other stations, it was possible to detect considerably higher OMC values at BB in summer and spring. This similarity between seasons at BB station might be explained by the already known nutrient enrichment of this station and consequently highest OMC in the sediment. On the contrary, at PF station the highest OMC values were found in the autumn, followed by winter, and it was possible to detect a notable influence of seasons in the organic matter content. This station is relatively confined and consequently less susceptible to freshwater discharges, however these seasons of higher precipitation might origin highest loads of water from agricultural runoff. Also, at BB and AR stations, the OMC values were similar at all seasons, which could be explained by the continued influence of the freshwater discharges from the rivers throughout the year.

Óbidos lagoon receives different types of discharges, such as waste water from treatment plants (WWTP), livestock effluents and agriculture runoffs (Malhadas *et al.*, 2014), but also industrial wastewaters from ceramic and metal industries (Pedro *et al.,* 2016). Freshwater inputs to this lagoon come from the two opposite branches, via *Cal* river and *Ameal* ditch. However, located between the two arms the *Arnóia* river contributes with about 90% of freshwater discharges into the lagoon (Malhadas *et al.*, 2009). These freshwater fluxes are responsible for the nutrient and contaminant loads from agriculture fields, domestic and industrial effluents discharges into the lagoon. As a result, both eutrophication (Pereira *et al.,* 2009b) and metals' accumulation (Mn, Fe, Cu, Cr, Ni, Co and Cd) (Pereira *et al.,* 2009c) were already reported in the lagoon.

All the studied metals were found in the Óbidos Lagoon during the studied period. In general, when detected, all the studied essential metals (Cu, Fe, Mn and Zn) were found in higher concentrations, dissolved and suspended in the water column. Both in the winter and/or autumn at BB and/or AR stations, which may be associated with the higher precipitation periods and consequently, higher water discharges from rivers and runoffs. In addition, all essential metals were found in the sediments in all seasons. However, statistically significant higher concentrations were found at BB in all seasons. Also, at PF were found higher metal concentrations in the autumn and winter, when compared with AR and CM stations. This may be related with the sediment composition at BB and PF stations, which are rich in silts and consequently contaminants tend to sink easily, as previously reported by Carvalho *et al*. (2005) in Óbidos Lagoon.

Additionally, the non-essential metals (Cd and Pb), analyzed in this study, were also found in the environmental samples. Although in lower concentrations compared with the essential metals, as expected. Both metals were found in the sediment and in the water column, however Pb was only found suspended. Contrary to essential metals, Cd concentrations in environmental samples were similar between seasons and stations. On the other hand, Pb concentrations were relatively high in the winter at all stations and in summer at BB station. Higher concentrations in the winter may be associated with the precipitation period and the higher concentrations observed in the summer were probably related to contamination by human activities.

As expected, different behaviors were observed in the essential metals studied. Suspended Cu was detected at all seasons in concentrations between 0.034 mg.L^{-1} ± 0.027 and 0.112 mg.L^{-1} ± 0.055 However, a pattern between seasons and stations was observed. In each station suspended Cu was only detected two times in two consecutive seasons. As mentioned before suspended copper in the autumn and winter may be related to the precipitation period. Also, the highest concentrations were found at AR and BB station and the statistical tests supported this. In the present study, the concentrations of Cu detected in the sediment were between 1.52 mg.kg^{-1} ± 0.62 and 55 mg.kg^{-1} ± 7.78. The highest concentrations were found at BB station in all seasons, which was expected in this station due its contamination level and the high organic level content. Also, higher Cu concentrations were found in autumn and winter at PF station, as compared to the other seasons. This may be explained by the increase of runoffs during the precipitation period, also the embayment characteristic of this station, and the high OMC values, which are known for an easily sinking of contaminants. In addition, the relatively higher Cu concentrations found suspended and in the sediment, in summer, may be a consequence of the human activities, a characteristic of this period. Pereira *et al.*, (2009a) studied the metal contamination at Óbidos Lagoon and its biochemical impact in a shore crab, in the autumn of 2005. Those authors reported Cu concentrations in the sediment of 56 mg.kg^{-1} in the entrance of BSB and 52 mg.kg^{-1} in the entrance of BB. Comparing to the present study, the BB and BSB stations were in the entrance of the arms, which mean before the BB and CM stations, respectively. In 2006, (Pereira *et al.*, 2009c) reported Cu concentrations in the sediment between 46 and 68 mg.kg^{-1} in the entrance of BSB and Cu concentrations between 41 and 83 mg.kg^{-1} in the entrance of BB. In general, in the present study similar values were found at BB station, but at CM station Cu concentrations in the sediment were clearly lower.

The element iron was detected in all seasons and stations in all environmental samples. Dissolved iron concentrations were between 0.012 mg.L^{-1} ± 0.010 and 0.059 mg.L^{-1} ± 0.000 and suspended iron concentrations were between 2.34 mg.L^{-1} ± 1.49 and 56.17 mg.L^{-1} ± 30.52. In general, higher concentrations were found in the water column in periods of higher precipitation and statistically significant differences were found in the dissolved iron in the winter and autumn. The sediment iron concentrations were between 1966 mg.kg^{-1} ± 330 and 58067 mg.kg^{-1} ± 19100, and the highest Fe concentrations were found at BB station compared with the other stations, which was corroborated by the statistical tests performed. The higher Fe concentrations at BB station may be associated with the relatively high organic matter content and the contamination level at this station. In general, iron concentrations were significantly higher in suspended matter, sediment and biological samples when compared with the other metals, and due to the natural abundance of this metal in the environment (Kabata-Pendias, 2011) these concentrations were expected.

The dissolved Mn was only detected at BB and AR stations and the concentrations were between 0.002 mg.L^{-1} ± 0.001 and 1.000 mg.L^{-1} ± 0.012. However, differently as dissolved Mn, suspended Mn in the water samples ranged between 0.18 mg.L^{-1} ± 0.13 and 4.05 mg.L^{-1} ± 0.57 and, as mentioned before, suspended manganese concentrations showed evidence of the influence of freshwater discharges from precipitation periods and Bonferroni test supported this., It is known that Óbidos lagoon is influenced by agriculture runoffs (Malhadas *et al.*, 2014; Pinto *et al.*, 2016) and BB and AR stations are directly influenced by freshwater river discharges. Consequently, the highest Mn concentrations in water column in these two stations were probably related to river inputs. The highest Mn concentrations in the water column were found in summer at BB station and the Bonferroni test supported this. Zhou *et al*. (2003) studied trace metal behavior in Conwy estuary (United Kingdom) and observed that dissolved manganese tended to decrease with salinity. Also, between salinities of 20 and 35, dissolved Mn concentrations were approximately between 0.1 µg.L^{-1} and 2.3 µg.L^{-1}. In the present study, dissolved Mn was only detected in sanities below 30 and the highest Mn concentration (1 mg.L^{-1}) was found in summer at BB station with a salinity of 22. Low salinities were also found in winter, however dissolved Mn was considerably lower than in summer. Due to the lower freshwater discharges in summer, the higher dissolved Mn concentrations at BB in this season, may be associated with the low water pH value (pH=5.3). Duinker *et al*., (1979) studied the manganese cycle in Rhine and Scheldt estuaries (Netherlands) and reported that after manganese deposition in sediment, and subsequent reduction, or once in contact with low pH water, Mn may return into the dissolved compartment within the water column. Pereira *et al.,* (2009a) in 2005 found dissolved Mn in water column in the entrance of both arms BSB and BB and the highest concentrations were 0.44 µM at BSB and 0.31 µM at BB. In this study, the Mn concentrations in water column were clearly higher. In the sediment, the manganese concentrations were between 3.33 $mg.kg^{-1}$ ± 2.72 and 926.48 $mg.kg^{-1}$ ± 279.12 and the highest concentrations were found at BB station in all seasons, as confirmed by the Tukey HSD test. Differently to other metals, manganese presence in the sediment was similar between different seasons. Pereira *et al.*, (2009a) also reported Mn concentrations in the sediment of 300 $mg.kg^{-1}$ at the entrance of BB and 286 $mg.kg^{-1}$ at the entrance of BSB. In 2006, Pereira *et al.*, (2009c) reported similar Mn concentrations at BB (between 269 and 301 $mg.kg^{-1}$) and at BSB (between 266 and 322 $mg.kg^{-1}$). In the present study, Mn concentrations were clearly higher at BB station and lower at CM station comparing with the previous concentrations reported by Pereira *et al.*, (2009a and 2009c).

Suspended zinc was only detected in winter and spring in concentrations between 0.006 $mg.L^{-1}$ ± 0.005 and 0.1777 $mg.L^{-1}$ ± 0.0997 and, as previously mentioned, this may be related with precipitation periods. According to Zhou *et al.*, (2003), suspended Zn concentration in Conwy estuary tend to decrease seawards, which means that suspended Zn decreases with higher salinities. In the present study, higher suspended Zn concentrations were found in salinities below 30. In the sediment samples the Zn concentrations were between 3.24 $mg.kg^{-1}$ ± 2.64 and 111.72 $mg.kg^{-1}$ ± 17.47 and the highest concentrations were found at BB station in the spring and summer, which was supported by the Bonferroni test. The higher Zn concentrations at BB station were probably associated with the already known contamination and the high organic matter content. The higher concentrations in the sediment at BB, in periods of lower precipitation characterized by lower water discharges (summer and spring) may be related with a lower water renewal and, consequently, an easily sinking of organic matter and contaminants. On the contrary, also at BB station, during the periods of higher precipitation the water renewal is higher (Malhadas *et al.*, 2010). Consequently, the lower concentrations in the autumn and winter could be related with contaminant resuspension, which is a common behaviour in estuaries (Amiard-Triquet *et al*., 2015). On the contrary, at PF station the highest concentrations were found in the autumn and winter, which could be related with runoffs from precipitation period. Also, Zn may had easily sunk at PF station due to sediment characteristics and its OMC. In addition, the undetected values at PF station in summer may be associated with metal resuspension from sediment, as previously observed in this eutrophic environment (Pereira *et al.*, 2009b). Pereira *et al.*, (2009a) in 2005, reported Zn concentrations in the sediment of 144 mg.kg^{-1} in the entrance of BB and 121 mg.kg^{-1} in the entrance of BSB. In the present study, similar concentrations were found at BB station, however at CM station Zn was not detected in the sediment.

The non-essential metals studied (Cd and Pb) were both found in the sediment and in water column. The higher concentrations in the winter of dissolved and suspended Cd obtained in the present study may be related to the precipitation period, and consequently the contaminant source could be mainly from an increase in the freshwater discharges. It is known that the free ionic form of Cd is able to form complexes with organic compounds and oxides and is not soluble above pH 7.5 (Pedro *et al*., 2013). In the present study, only in the summer at BB and PF stations the water pH was below 7.5, which suggests that, in general, Cd was mainly from recent freshwater discharges. Contamination in the autumn and winter is associated with the precipitation period and in the other seasons the contamination was probably from sediment resuspension. Also, Cd contamination might be from the increasing of human activities and higher domestic discharges through Óbidos Lagoon, typical of summer and spring seasons. The highest concentrations were found in the winter and spring at BB and AR stations. In 2005, Pereira *et al.*, (2009a) detected dissolved Cd at Óbidos Lagoon. In the entrance of BB the highest concentration of dissolved Cd reported was 0.17 nM and in the entrance of BSB was 0.080 nM. In addition, Pereira *et al.*, (2009c) in 2006, also reported dissolved Cd concentrations between 0.07 and 0.29 nM in the entrance of BB and concentrations between 0.05 and 0.19 nM in the entrance of BSB. In the present study, the dissolved Cd concentrations were considerably higher at BB station and similar at CM station (BB: 0.099 - 1.173 nM; CM: 0.056 - 0.228 nM), comparing to the entrances of BB and BSB, respectively. Pedro *et al.*, (2016) evaluated Óbidos Lagoon contamination by Cd between 2009 and 2010 (S_4, S_5, S_6 and S_7 stands for BB, AR, CM and PF, respectively) and the highest suspended Cd concentrations were found at BB station in the winter of 2010 (approximately 1.05 mg.L^{-1}), followed by AR station in the autumn of 2009 (0.90 mg.L^{-1}), CM station in winter of 2010 (0.80 mg.L^{-1}) and PF station in the autumn of 2009 (0.85 mg.L^{-1}) and in the summer 10 (0.60 mg.L^{-1}). In the current study, the suspended Cd concentrations were lower (between 0.009 and 0.678 µg.L^{-1}). However, it should be noticed that at BB and AR stations values above the established limited by the Decree-Law n. 103/2010 were found. These results suggest that Óbidos Lagoon is still being contaminated by cadmium via freshwater discharges. In the sediment the concentrations of Cd obtained were between 0.0002 mg.g^{-1} ± 0.0001 and 143.66 mg.g^{-1} ± 117.29 and the highest concentrations were found in the spring at PF and BB stations. Cd adsorption and desorption rates are rapid on particles of clay (Eisler, 2007). As mentioned before, the sediment composition at BB and PF stations is mainly silt and clay. Therefore, this might explain the highest Cd concentrations in sediment in the spring at BB and PF and the non-detection at AR and CM stations. It is known that pH influences metals behaviour, and Cd is not soluble above pH 7.5, while a lower pH promotes Cd remobilization from sediments (Pedro *et al*., 2016). Like such, the higher Cd concentrations in the sediment in spring could be also related to the highest pH in the water column in this season (pH 8.9). In 2005, Pereira *et al.*, (2009a) analysed Cd concentrations in the sediment from Óbidos Lagoon. In the entrance of both arms, BB and BSB, these authors found concentrations of 0.19 mg.kg^{-1} and 0.17 mg.kg^{-1}, respectively. In 2006, Pereira *et al.*, (2009c) reported concentrations between 0.15 and 0.29 mg.kg^{-1} and 0.13 and 0.23 mg.kg^{-1} in the entrances of BB and BSB, respectively. Comparing the present study with both studies of Pereira *et al.*, (2009a,c), in 2019 Cd concentrations were considerably higher at BB station than in 2005 and 2006. Pedro *et al.*, (2016) also reported Cd concentrations in the sediment from Óbidos Lagoon at BB station in the spring 2010 (7.0 mg.kg^{-1}); at AR station in the spring 2009 (3.5 mg.kg^{-1}) followed by winter 2009 (2.5 mg.kg^{-1}); and at PF station in the winter 2010 (3.0 mg.kg^{-1}). In the present study, Cd concentrations in the sediment were clearly higher in spring 2019 at BB (60 mg.kg^{-1}) and at PF (144 mg.kg^{-1}) stations. In conclusion, the Cd in water column appears to be lower compared with past years, however that Cd in water column may be associated with recent discharges into the lagoon (Pedro *et al.*, 2016). On the contrary, the Cd in the sediment was considerably higher in the present study compared with such previous studies. These results and the evidence that Cd in the sediment is a reflex of long-term pollution (Pedro *et al.*, 2016), clearly suggesting that cadmium is still a prevalent contaminant in Óbidos Lagoon.

Lead concentrations suspended in the water column samples were between 0.57 µg.L^{-1} ± 0.46 and 24.70 µg.L^{-1} ± 10.12 and the Pb concentrations found at BB station were considerably higher in the winter compared with the other seasons and stations. Also, similar concentrations were found at AR, CM and PF in the autumn and winter. In general, Pb solubility is low in water, except in areas of local point source discharges (Eisler, 2007). Then, the higher suspended Pb concentrations in the winter and autumn were probably related to freshwater discharges originate from precipitation period and water runoff from agricultural fields. Only at BB in winter the lead concentrations were considerably higher comparing to the established limits by the Decree-Law n. 103/2010. In the sediments, the Pb concentrations were between 2.08 mg.kg^{-1} ± 0.84 and 77.14 mg.kg^{-1} ± 2.70 and the highest Pb concentrations were found at BB station in the summer compared to the other stations and seasons. Also, relatively high Pb concentrations were found in the sediment in the winter at all stations, as highlighted by the results of the Bonferroni test. Differently from the other metals, Pb was found in the sediment in similar concentrations in all seasons except for BB in summer, which were considerably higher. Aydin-Onen *et al.* (2015) associated higher Pb concentrations in the sediment to human activities, then higher Pb concentrations in summer may be associated with the increase of human touristic and fisheries practices in the Lagoon in this season and, consequently, an increase in the number of pollution sources. Carvalho *et al.* (2011) analysed Pb concentrations in the sediment from Óbidos Lagoon, in 2006, and found Pb concentrations between 35 and 52 mg.kg^{-1} at the entrance of BB arm, and 33 and 49 mg.kg^{-1} in the entrance of BSB arm. BB and BSB stations were located before the BB and CM stations, respectively in the present study, and in 2018 higher concentrations (77 mg.kg^{-1}) were found at BB as compared to the previous results in 2006. These suggests that Óbidos Lagoon is still being similarly contaminated by lead since 2006 until the present days.

In general, dissolved metals in the water column are usually associated with recent inputs (Wong *et al*., 2007), therefore it is clear that all stations suffer higher contamination in periods of precipitation with higher concentrations at BB station, followed by AR station, which are highly influenced by freshwater waterflows compared to CM and PF stations. On the contrary, the estuarine sediments, known as the main reservoir of contaminants (Ribeiro *et al*., 2018), consequently metal concentrations in sediments are strong indicators of the continued effect of metal contamination. All metals were found in the sediment, in almost all stations. However, all the studied metals were found in higher concentrations at BB station, which was expected due to the already known high metal contamination level (Pereira *et al*. 2010).

In the present study, several correlations between the contaminants and the environmental samples were found. It is known that metals behaviour is related with the interaction with other multiple compounds, including other metals (Machado *et al.*, 2016). Spearman correlations results showed very strong positive correlations between copper and iron in the sediments, as well as between iron and manganese concentrations. Also, strong positive correlations were found for the concentrations in the sediments between copper and manganese, copper and zinc, iron and zinc, and manganese and zinc. These correlations suggest that these metals were probably originated from the same source, similarly as Aydin-Onen *et al.* (2015) reported for Cu and Zn. Also, positive modest correlations were found between suspended copper and suspended iron and between suspended copper and suspended manganese. In addition, a strong positive correlation was found between suspended iron and suspended manganese and a modest positive correlation was found between dissolved iron and suspended lead, which might suggest that these suspended metals were also from the same source like reported in Aydin-Onen *et al.*, (2015) for Cu and Zn in the sediment.

The contamination was also evaluated in *H. diversicolor* and all the metals were found in the organisms in the following order: Fe>Zn>Mn>Cu>Pb>Cd. In addition, the concentration and bioaccumulation factors in *H. diversicolor* allow to evaluate metal contamination and it is known that bioaccumulation of metals by the organism, via sediment, occurs if the BAF value is above 1 (Aydin-Onen *et al.,* 2015). In general, BAF values may be ranked in the following order in *H. diversicolor*: Cd>Zn>Cu>Pb>Mn>Fe. However, all BAF values for Fe were lower than 1 and in the other metals, the bioaccumulation did not occur in all seasons and sampling sites. As mentioned before, *H. diversicolor* may also act as a filter-feeder collecting particles from water. Then concentration factors were also calculated. In general, CF values may be ranked in the following order in *H. diversicolor*: Zn>Cu>Fe>Mn>Cd>Pb.

Cu concentrations in *H. diversicolor* were between 0.30 mg.kg^{-1} ± 0.27 and 47.05 mg.kg^{-1} ± 8.23 and were mainly found at AR station, and at CM station it was found in lower concentrations. To support this, Bonferroni test results showed significant differences. It is known that *H. diversicolor* are capable of tolerating copper (Mouneyrac *et al.*, 2003) and it is believed that copper accumulation in polychaetes its related with the nature of the surrounding substrate (Eisler, 2010). In general, this behaviour was also observed in this study.

Copper BAF values were between 0.06 and 7.74 and were obtained in all seasons and sampling stations, except in the summer at CM station and in the winter at BB station. It should be noticed that only at AR and CM stations were found direct evidences of bioaccumulation via sediment. The highest Cu BAF values were found at AR station, with the exception of spring when higher values were obtained at CM station, as corroborated by the results of Bonferroni test. In addition, spearman test showed a very strong correlation between Cu BAF and the concentration in the organism, which was expected due to their relationship. Also, it might confirm the influence of Cu concentrations in the sediment in the bioaccumulation in the organisms, which was expected due to relatively high Cu concentrations in the sediment and organisms. Frangipane *et al.*, (2005) studied metals bioaccumulation in populations of *H. diversicolor* from the Lagoon of Venice (Italy) and found copper concentrations between 15.08 mg.kg^{-1} and 39.44 mg.kg^{-1} in the organisms. In the present study, similar concentrations to the ones reported in Venice Lagoon were only obtained in the organisms from AR and BB stations. Those authors also reported Cu BAF from the organisms from Venice

Lagoon between 0.72 and 14.00, which were relatively higher compared with the present study. Idardare *et al.* (2008) studied metal bioaccumulation in *H. diversicolor* from two distinct lagoons (Oualidia and Khnifiss, Morocco) during an year and reported Cu concentrations in the organisms between 6.8 mg.kg^{-1} ± 2.5 (Oualidia) and 8.8 mg. kg^{-1} ± 3.8 (Khnifiss). Those authors also reported Cu BAF values between 0.4 ± 0.2 (Oualidia) and 0.5 ± 0.3 (Khnifiss), which are considerably lower compared with some values obtained in the present study. It appears that the organisms from Óbidos Lagoon present a higher Cu bioaccumulation ability when compared with Oualidia and Khnifiss Lagoons organisms, while the ragworms from Venice Lagoon bioaccumulate Cu more easily than the ones from Óbidos Lagoon.

Concentration factors were also possible to calculate in the organisms from all stations and in different periods of sampling, with values between 4.4 L.kg^{-1} ± 4.1 and 1381 L.kg^{-1} ± 242. Similar as BAF values, the highest Cu CF values were also found in animals from AR station, however only in summer and autumn, which suggest that the Cu concentrations in the organisms at AR station were also bioaccumulated via Cu concentrations in the water column. To support this, Bonferroni test showed significant differences in the organisms from AR station and spearman test also showed a modest correlation between Cu CF values and the Cu concentrations in the organisms.

In *H. diversicolor* Fe was detected in all studied periods, with similar concentrations at all stations and seasons (between 204 mg.kg^{-1} ± 55 and 772 mg.kg^{-1} ± 370). In addition, iron BAF values were obtained in all seasons and sampling stations and ranged between 0.008 ± 0.001 and 0.195 ± 0.017. The highest Fe BAF values were found at CM station. However, Fe bioaccumulation via the sediment was not observed since none of the values were above 1. The lower BAF values for Fe compared with the other metals and the abundance of Fe in the sediment and biological samples suggest that the accumulation of iron in *H. diversicolor* is easily regulated by the organism (Bryan & Hummerstone, 1971). In other words, *H. diversicolor* appears to accumulate iron if it is necessary for the organisms' metabolism. Additionally, this organism presents haemoglobin (Demuynck & Dhainaut-Courtois, 1993) which is also an evidence of iron control by the organism. It was possible to calculate CF values in the organisms from all sites and periods of sampling and the values were between 6 L.kg^{-1} ± 2 and 252 L.kg^{-1} ± 121. The highest CF values were found in organisms from CM station in the winter and spring and from BB station in the spring, as evidenced by the Bonferroni test results. Contrary to BAF values, the Fe CF values suggest that the bioaccumulation of Fe in *H. diversicolor* was probably mainly influenced by the iron available in the water column.

The Mn concentrations in the organisms were between 2.5 $mg.kg^{-1}$ ± 2.3 and 66 $mg.kg^{-1}$ ± 4.8. The metal Mn was only detected in *H. diversicolor's* tissues at all stations in spring, and in higher concentrations than at autumn and winter. Also, in spring, higher concentrations were found at AR, followed by PF, as supported by the Bonferroni test. Mn BAF values ranged between 0.050 ± 0.009 and 2.066 ± 0.178, but the only relevant values (BAF>1) were obtained in the spring at CM and PF stations. Since high Mn concentrations were obtained in the environmental samples in the spring, these results were expected. According to Bryan & Hummerstone (1973), manganese appears to occur in the organism in two pools. The slowly exchanging pool is of the order of 10 $mg.kg^{-1}$ (dry weight) and it seems to include the Mn which is biochemically essential. There is also a more readily exchangeable pool, and its amplitude depends on several factors, such as the concentration of manganese in the interstitial water and its salinity. Then it was expected that *H. diversicolor* bioaccumulate Mn easily. In the present study, only in the spring were found concentrations above 10 $mg.kg^{-1}$, which suggests that *H. diversicolor* easily regulates Mn bioaccumulation. Frangipane *et al.*, (2005) reported Mn concentrations in *H. diversicolor* from Venice Lagoon between 5.47 $mg.kg^{-1}$ and 11.28 $mg.kg^{-1}$. In the present study, relatively higher Mn concentrations were found in the spring when compared with Venice Lagoon. Those authors also reported Mn BAF values between 0.05 and 0.13, which are relatively smaller than the ones observed in the present study. This suggests that the organisms from Óbidos Lagoon appear to have a higher Mn bioaccumulation ability than the organisms from Venice Lagoon. As for the concentration factor, Manganese CF values ranged between 2.4 $L.kg^{-1}$ ± 2.2 and 260 $L.kg^{-1}$ ± 19 and were considerably high in spring compared with the other seasons, as evidenced by the Bonferroni test results. Also, similarly as for Mn BAF values, Mn CF values in winter were only obtained in the organisms from CM station and in autumn only from CM and PF stations.

Zinc in *H. diversicolor* was detected in all seasons and stations in relatively high concentrations (53 $mg.kg^{-1}$ ± 13 to 133 $mg.kg^{-1}$ ± 24). However higher concentrations were found in the animals from AR station, followed by PF and BB stations, as supported by the Bonferroni test. Zinc is an essential metal used as co-factor for enzymes in this species and Zn concentrations can be regulated by the organisms (Gomes *et al.*, 2013). Therefore, the relatively high concentrations obtained were expected. Zinc uptake by *H. diversicolor* usually increases with highest Zn concentrations in the sediment, lower salinities, and elevated temperatures (Eisler, 2010). However, in the present study this behaviour was not demonstrated. Zinc BAF values ranged between 0.67 ± 0.13 and 32.93 ± 5.94. It was only possible to calculate Zn BAF values for all seasons at AR station and with evidence of bioaccumulation (BAF>1). Also, at PF station in both analysed seasons (autumn and spring) it was observed bioaccumulation, while at BB station this was observed only in autumn. The highest Zn BAF values were found in the winter at AR station and in the spring at AR and PF stations, which was supported by the Bonferroni test results. Tolerance to zinc in sandworms may be a result of acclimatization or genetic adaptation (Eisler, 2010). The highest and the similar Zn BAF values at AR, CM and PF stations compared with BB station may be associated with the highest sediment contamination at BB station, and consequently the organisms from BB station are probably most tolerant to Zn. Idardare *et al.*, (2008) studied metal bioaccumulation in *H. diversicolor* from two distinct lagoons (Oualidia and Khnifiss) and found Zn concentrations in the organisms of 115 mg.kg^{-1} ± 30 and 94 mg.kg^{-1} ± 44, which are similar to the concentrations obtained in the present study. Those authors also reported Zn BAF values between 1.1 ± 0.6 and 1.4 ± 0.8, considerably lower when compared to most Zn BAF values obtained in this study. These results suggest that organisms from Oualidia and Khnifiss Lagoons are probably more tolerant to zinc than the organisms from Óbidos Lagoon. Zn CF values were the highest compared with all the other metals. However, it was only possible to calculate Zn CFs in the winter, at BB and AR stations, and in the spring, in all stations except BB. The highest values were found in the organisms from AR station, followed by PF station. These results suggest that zinc concentrations in the organisms were bioaccumulated in the summer and autumn mainly via sediment and in the winter and spring mainly via the water column. Gomes *et al.* (2013) studied metal contamination (Cd, Cu, Ni, Pb and Zn) in *H. diversicolor* from *Ria Formosa* in autumn 2006 and spring 2007. Only in one sampling site, in the spring were found significantly higher Zn concentrations. Also, Durou *et al.* (2005) studied the zinc lethality in *H. diversicolor* from two different estuaries (Authie and Seine, France) and concluded that organisms from the highest polluted estuary (Seine) had higher tolerance to zinc.

Cu, Fe, Mn and Zn are essential metals and inherent tolerance of *H. diversicolor* to Cu and Zn is already known (Scaps, 2002). In addition, the regulation by the organism is suggested (Bryan & Hummerstone, 1971) and this could explain the relatively low BAF and CF values for Cu, Fe and Mn. Fe was found in higher concentrations in the organisms than Zn, however the Zn BAF and CF values were higher compared to Fe and the other essential metals. These results probably mean that organisms from Óbidos Lagoon are less tolerant to Zn differently as to the other essential metals. Less tolerance to Zn was not expected due to previous reports by different authors.

As mentioned before, metals interact with each other. A modest positive correlation was found between Fe CF and Mn concentrations in the organisms which suggests that Mn concentration in the organism influence the bioaccumulation of Fe via water column. In addition, a modest positive correlation was found between Zn CF values and Mn BAF values, which suggests that Zn bioaccumulation via water column influence Mn bioaccumulation via sediment and/or vice versa. Also, a modest positive correlation was found between Zn and Fe BAF values which probably means that Zn and Fe bioaccumulation in the organisms via sediment influence each other.

Non-essential metals, such as Cd and Pb, are known to be toxic for all living organisms, and *H. diversicolor* are known to be capable of tolerating these metals. In the present study, Cd concentrations ranged between 0.0010 $mg.kg^{-1}$ ± 0.0006 and 0.4486 $mg.kg^{-1}$ ± 0.1726, with highest concentrations in the organisms from AR station, followed by BB station, as confirmed statistically by the Tukey HSD test. Also, Cd BAF values were between 0.002 ± 0.001 and 1617 ± 747 and were only calculable for the animals from BB and CM stations, with bioaccumulation observed only in winter and summer, at BB and CM stations, respectively. However, the highest Cd BAF values were found at BB station in the winter and the Bonferroni test results supported this. In their study, Idardare *et al.* (2008) reported Cd concentrations in the organisms between 0.09 $mg.kg^{-1}$ ± 0.06 and 1.0 $mg.kg^{-1}$ ± 0.2 at Oualidia and Khnifiss Lagoons which were relatively higher compared to the concentrations obtained at Óbidos Lagoon. In addition, those authors found Cd BAF values between 0.2 ± 0.2 and 2.5 ± 1.2, which are considerably lower compared to the ones obtained in this study. These results suggest that organisms from Oualidia and Khnifiss Lagoons are probably less susceptible to Cd when compared with the organisms from Óbidos Lagoon. Concerning Cd CF values, they were obtained in all seasons and sampling sites, except for CM and PF stations in spring. Cd CF values ranged between 0.17 $L.\mu g^{-1}$ ± 0.10 and 30 $L.\mu g^{-1}$ ± 8. The highest Cd CF values were always found at AR station and, with regards to seasons, the highest values were found in autumn, as evidenced by the Bonferroni test results. Mouneyrac *et al.* (2003) suggests that reduced Cd accumulation by *H. diversicolor* may be associated with the high Zn bioavailability and consequently higher tolerance to Cd, which was consistent with the results of the present study, where it was found relatively high Zn concentrations in the organisms and low Cd BAF values, except for BB station in the winter. Also, it is believed that Cd concentration accumulated in *H. diversicolor* is proportional to the surround environment (Bryan 1976; Bryan *et al*. 1980; Amiard *et al*. 1987 *in* Berthet *et al*., 2003). In addition, a very strong positive correlation was found between the cadmium concentration factor and the Cd concentration in the organism which suggests that bioaccumulation in the organism was also influenced by suspended and dissolved Cd in the water column and not only via sediment.

Lead was detected in the organisms from all sampling stations and studied periods, with concentrations ranging between 0.061 mg.kg^{-1} ± 0.055 and 8.03 mg.kg^{-1} ± 2.09. In general, the highest concentrations were found at AR station, but in winter Pb concentrations were relatively higher at BB station, which was supported by the Bonferroni test. Pb BAF values were obtained in all seasons and stations and values were between 0.02 ± 0.01 and 3.12 ± 0.81. However, the only moment that bioaccumulation was verified was in the autumn at AR station, as confirmed by the Bonferroni test results. Frangipane *et al.*, (2005) reported Pb concentrations in the organisms from Venice lagoon between 0.68 mg.kg^{-1} and 1.54 mg.kg^{-1}, and Pb BAF values between 0.01 and 0.06. These results were considerably lower compared to the Pb concentrations and BAF values obtained in this study, which suggest a higher contamination by Pb in Óbidos Lagoon compared to Venice Lagoon. Pb CF values were relatively low, ranging between 0.065 L.µg^{-1} ± 0.058 and 1.72 L.µg^{-1} ± 0.45 and, similarly to BAF values, the highest CF value was found at AR station in the autumn, as evidenced by the Bonferroni test. In addition, spearman test showed a very strong positive correlation between bioaccumulation factor and the Pb concentration in the organism. This result suggests that the accumulation in the organisms was strongly influenced by the metal concentrations in the sediment. According to Ruus *et al.*, (2005), ragworms are known to absorb most of Pb by sediment ingestion. Also, a modest positive correlation was found between suspended lead and lead concentrations in the organism and between Pb CF values and Pb concentrations in the organisms, which suggests that suspended Pb may have also influenced the bioaccumulation of this metal in the organisms.

It is known that trace metals interact with each other (Machado *et al.*, 2016) and spearman test showed several correlations between cadmium and lead. A modest positive correlation was found between cadmium concentration factor and the lead concentrations in the organisms. Also, a modest positive correlation was found between lead bioaccumulation factor and the cadmium concentrations in the organisms. A modest positive correlation was found between lead bioaccumulation factor and cadmium concentration factor and a modest positive correlation was detected between cadmium concentration in the organisms and lead concentrations in the organisms. These results suggest that Cd and Pb bioaccumulation in the organisms were clearly related and might be from the same source, similar as Aydin-Onen *et al.*, (2015) reported for Cu and Zn.

To evaluate the health conditions of *H. diversicolor* populations, biometrical analyses were performed. As mentioned before, the L3 length, body size and wet weight are indicators of populations status (Durou *et al.*, 2008). In general, at AR and BB stations all biometrical parameters (L3 length, body size and wet weight) were lower than at CM and PF stations. Also, for all stations, the L3, body size and wet weight values were higher in the spring, except for body size at AR station. In all seasons and biometrical parameters, significant differences between BB station and CM and PF stations were detected. In brief, higher metal contaminations were associated with BB and AR stations. Therefore, organisms from these stations were high susceptible to stress. It is evident that at CM and PF stations *H. diversicolor* individuals had longer L3 lengths, bigger body sizes and were heavier, and consequently organisms appear to be in a better health condition as compared to the animals from BB and AR stations. In order to evaluate and compare the conditions of two estuaries (Authie and Seine, France), Durou *et al.*, (2008) studied different biometric variables in *H. diversicolor* and found lower health conditions in the organisms from the most polluted estuary (Seine). Additionally, in the present study, spearman test showed a positive modest correlation between L3 and body size and between L3 and wet weight. Also, the parameters body size and wet weight showed a positive strong correlation. These correlations results were expected and support the already known relationships between those biometrical parameters.

The metals Cu, Fe, Mn and Zn are essential elements for practically all living organisms, so it was expected that biometrical parameters may be related to these metals. Spearman correlation showed several positive modest correlations with the metal Mn: (i) between L3 and Mn concentration in *H. diversicolor;* (ii) between Mn BAF values and L3 length; (iii) between Mn CF values and L3 length and (iv) between body size and Mn BAF. These correlations suggest that Mn is clearly related to the organism growth. In addition, a modest positive correlation was found between wet weight and Cu BAF values which also suggest an influence of Cu in the organism's growth.

Non-essential metals, such as Pb and Cd are associated to stress in the organisms, however spearman correlation only showed an inverse modest correlation between wet weight and Pb concentration in the organism, which suggests that Pb bioaccumulation has a negative influence on the organisms' growth. Lighter organisms appears to have higher Pb concentrations which indicates that organisms reserves were lower in order to response to Pb accumulation. Garcês & Costa, (2009) reported that Pb concentration in the organisms *Marphysa sanguinea* (Montagu, 1813) is influenced by the worm's weight, with a tendency to an inverse relationship. In the present study, the relatively lower Cd concentrations in the organisms might be related to the known adaptive strategies of these worms. These organisms are known by secreting higher amount of mucus in response to higher metals concentrations, which help in reducing metal availability for uptake, similar as Mouneyrac *et al.*, (2003*)* reported for Ag and Cu.

In addition, spearman test also showed significant correlations between the non-essential metal Pb and the essential metals Fe and Mn. Modest negative correlations were found between Mn and Pb concentration factors; also, modest negative correlations were found between Pb concentration factors and Mn bioaccumulation factors; and between Pb and Fe concentration factors. These results suggest that Mn clearly affects positively the organisms' growth and, on the contrary, the element lead is associated to less organism growth.

Beside the ones mentioned above, there are also other different methods that allow to evaluate the health of organisms from different populations, such as the lipid content. In general, the lipid content was higher at CM station compared with AR station, except for spring. It is expected that stations with higher contaminants concentrations revealed lower lipid content. In 2002, Durou *et al*., (2005) studied the lipid content of *H. diversicolor* from two distinct estuaries (Authie and Seine) on a seasonal basis. Those authors reported lower lipid content in organisms from the Seine estuary, which is known to be more contaminated then the Authie estuary. Also, the non-essential metal Pb was the only metal that appeared to influence lipid content in the present study. A modest negative correlation was found between Pb concentration in the sediment and the lipid content, which probably means that the Pb available in the sediment affects negatively the lipid content in the organisms. In 2002 and 2004, Durou, *et al.*, (2007) studied biomarkers and chemicals in *H. diversicolor* in order to evaluate the conditions of two different estuaries (Authie and Seine). Those authors observed that the most contaminated estuary (Seine) is clearly high correlated with metals than the less contaminated one (Authie), which is most correlated with the energy reserves.

In brief, and as a whole, the environmental and biological samples analysed in the present study suggest that *Barrosa*'s Branch, and the *Arnóia* and *Real* rivers stations presents higher metals concentrations when compared with *Poça das Ferrarias* and *Covão dos Musaranhos* stations.

5. Conclusion

The current study allowed to conclude that during the four seasons, the parameters water temperature and salinity were different between the sampling stations, showing a clear seasonal variation of these parameters and a clear tidal effect in *Poça das Ferrarias* and *Covão dos Musaranhos*.

The metal copper was obtained in practically all biological and environmental samples except for the dissolved fraction. In general, the highest concentrations suspended in water column were associated with precipitation period and with touristic activities during summer. Also, in the same period, considerably higher copper concentrations in the sediment at *Poça das Ferrarias* were associated with the high organic matter content. Higher copper concentrations at *Barrosa's B*ranch in summer were associated with human activities.

The element iron was detected in all environmental and biological samples. The highest concentrations in water column were associated with precipitation period. Considerably high iron concentrations in the sediment compared with the other metals were associated with the abundance of this metal. Also, iron in sediment reflects the metal accumulation at *Barrosa*'s Branch, the highest iron concentrations in the sediment were associated with the high organic matter content and the already known metal accumulation of this station.

The metal manganese was sporadically detected, dissolved in the water column and in biological samples, but also was detected in practically all suspended and sediment samples. The manganese presence in water column was associated with seasonal fluctuations (precipitation) and salinity but also appears to be influenced by other water physicochemical parameter such as pH, which seems to influence manganese resuspension in summer. Manganese presence in the sediment only reflected the already known accumulation at *Barrosa*'s Branch.

The metal zinc was detected in all biological samples and in the sediment in practically all stations and seasons. Also, zinc was not detected dissolved in water column and in the suspended fraction it was only observed in two seasons. The suspended zinc was associated with seasonal fluctuations of precipitation and with salinity. Higher zinc concentrations in the sediment at *Barrosa*'s Branch were associated with the already known metal accumulation, lower water renewal and metals resuspension. The higher zinc concentrations in the sediment at *Poça das Ferrarias* were associated with the high organic matter content, also with seasonal fluctuations (precipitation) and with metal resuspension.

Metal contamination with cadmium was detected in the water column, sediment and in the organisms. The Cd concentrations in a few water samples were above the recommended limit by the law. Contamination by cadmium was associated with the seasonal fluctuations (precipitation) and the human activities. In addition, the results suggest that the pH is an important parameter in Cd accumulation in the environment. In general, cadmium remains an element of great concern in Óbidos Lagoon contamination.

Contamination with lead was detected in all biological and sediment samples, and also in several suspended samples. Only at *Barrosa*'s Branch in winter the lead concentration was considerably higher comparing to the established limits by the law. Contamination by suspended lead was associated with the seasonal fluctuations of precipitation and the highest concentrations in the sediment at *Barrosa*'s Branch were associated with human activities. It is evident that contamination by lead remains a great concern in Óbidos Lagoon.

The physiological responses of *H. diversicolor* reflected a spatial and seasonal fluctuation during the study period. Furthermore, *H. diversicolor* is a good indicator of the environmental fluctuations showing bioaccumulation behaviour for all studied metals (Cu, Fe, Mn, Zn, Cd and Pb). The bioaccumulation was manly observed for the essential metals iron and zinc, and for the non-essential metal cadmium. The results suggest that bioaccumulation of all metals occurs in the polychaete via sediment and via water column, except for iron which appears to bioaccumulate preferentially just via water column.

In brief, *H. diversicolor* revealed to be a good bioindicator of the fluctuations in Óbidos Lagoon. With respect to metal contamination, this organism was clearly affected by the different disturbances occurring on the environment, as demonstrated by the different populations from different sampling stations on the present study.

6. Future perspectives

In the development of this study, *Hediste diversicolor* demonstrated to be a good bioindicator species of spatial metal contamination in the Óbidos lagoon, similarly as referred on other studies. However, different approaches can be performed to continue the study on metal contamination in this lagoon or other estuarine environment using *H. diversicolor*:

- Evaluate other physiological parameters to assess population health, such as number and diameter of oocytes or the number of segments.
- Monitoring the bioaccumulation via sediment and via water column of metals Fe, Cu, Mn, Zn, Cd and Pb at laboratory controlled conditions to better understand the relationships between these metals in *H. diversicolor* and its bioaccumulation.
- Also, in laboratory monitored conditions, evaluate the organism tolerance to these metals to better understand the differences between levels of contamination in the different sites at Óbidos Lagoon.
- Continued monitoring metal accumulation in the environment and in *H. diversicolor* populations from the same stations at Óbidos Lagoon in order to assess contamination evolution.
- Investigate the correlations between the different metals and the bioaccumulation and concentration factors.

Considering these approaches and the *H. diversicolor* characteristics, there are numerous of possible studies that may contribute to a better understanding of the relationship between the metal contamination on the environment and the bioaccumulation abilities of these polychaetes. Therefore, *Hediste diversicolor* could be used as a regular bioindicator in environmental monitoring studies in Óbidos Lagoon and other estuarine environments.

7. References

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8. Attachments

Attachment 1 – Two-Way ANOVA and post-hoc test results for organic matter content (OMC) considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only the significant results were represented (ρ -value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
OMC				
Seasons*Stations	9	0.000	2.361	0.038

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
OMC			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	BB and AR	0.001
		BB and CM	0.000
		BB and PF	0.001
	Autumn	BB and AR	0.000
		AR and PF	0.000
		CM and PF	0.000
	Winter	BB and AR	0.005
		AR and PF	0.003
	Spring	BB and AR	0.005
		BB and CM	0.014
		BB and PF	0.018
		Comparison:	
	PF	Summer and Autumn	0.000
		Summer and Winter	0.004
		Autumn and Winter	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.009

Attachment 2 – Two-Way ANOVA and post-hoc tests results for metal concentration dissolved in water column considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ -value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
[Fe]				
Seasons*Stations	9	0.000	2.361	0.038
[Mn]				
Seasons*Stations	9	0.166	29.884	0.000

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
[Fe]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	AR and PF	0.008
		Comparison:	
	BB	Winter and Spring	0.003
	AR	Summer and Winter	0.003
	PF	Autumn and Winter	0.008

		Autumn and Spring	0.008
[Mn]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
		Comparison:	
	BB	Summer and Autumn	0.000
		Summer and Winter	0.000
		Summer and Spring	0.000

Attachment 3 – Two-Way ANOVA and post-hoc test results for suspended metals considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ -value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
[Cu]				
Seasons*Stations	9	0.005	2.808	0.016
[Mn]				
Station	3	8.041	4.356	0.012
[Pb]				
Season	3	259.704	6.303	0.002

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
[Cu]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	AR and PF	0.029
	Winter	BB and AR	0.021
		BB and CM	0.021
		Comparison:	
	BB	Summer and Winter	0.021
		Winter and Spring	0.021
	AR	Autumn and Winter	0.029
		Autumn and Spring	0.029
[Mn]			
Stations	Tukey HSD	**Comparison:**	
		BB and CM	0.007
		BB and PF	0.005
[Pb]			
Seasons	Tukey HSD	**Comparison:**	
		Summer and Winter	0.006
		Autumn and Winter	0.030
		Winter and Spring	0.004

Attachment 4 – Two-Way ANOVA and post-hoc tests results for metal concentration in sediment samples considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ -value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
[Cu]				
Seasons*Stations	9	199.341	3.151	0.008
[Fe]				
Stations	3	4100230396	29.000	0.000
[Mn]				
Stations	3	1134442.160	35.630	0.000
[Zn]				
Seasons*Stations	9	681.187	3.844	0.002
[Pb]				
Seasons*Stations	9	1019.405	38.516	0.000

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
[Cu]			
Stations*Stations	Bonferroni	**Comparison:**	
	Summer	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
	Autumn	BB and AR	0.006
		BB and CM	0.000
	Winter	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.048
	Spring	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
		Comparison:	
	BB	Summer and Autumn	0.005
	PF	Summer and Winter	0.029
[Fe]			
Stations	Tukey HSD	**Comparison:**	
		BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
[Mn]			
Stations	Tukey HSD	**Comparison:**	
		BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
[Zn]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
	Autumn	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.002
		CM and PF	0.038
	Winter	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
	Spring	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000

		Comparison:	
	BB	Summer and Autumn	0.012
		Summer and Winter	0.001
		Autumn and Spring	0.025
		Winter and Spring	0.003
	PF	Summer and Autumn	0.038
[Pb]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
		Comparison:	
	BB	Summer and Autumn	0.000
		Summer and Winter	0.000
		Summer and Spring	0.000
		Autumn and Winter	0.048
	AR	Autumn and Winter	0.001
	CM	Autumn and Winter	0.004
	PF	Summer and Winter	0.000
		Autumn and Winter	0.000
		Winter and Spring	0.002

Attachment 5 – Two-Way ANOVA and post-hoc tests results for metal concentration on the organisms *H. diversicolor* considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ - value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA

Source of variation	df	MS	F-statistic	p-value
[Cu]				
Seasons*Stations	6	957.813	3.404	0.004
[Mn]				
Seasons*Stations	6	951.018	9.888	0.000
[Zn]				
Seasons*Stations	6	12199.31	3.716	0.002
[Cd]				
Stations	3	0.593	5.455	0.002
[Pb]				
Seasons*Stations	6	59.042	2.188	0.049

Post-hoc tests

Source of variation	Test	Condition	p-value
[Cu]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	AR and CM	0.000
	Autumn	AR and CM	0.010
		Comparison:	
	AR	Summer and Autumn	0.044
		Summer and Winter	0.000
		Summer and Spring	0.000
		Autumn and Spring	0.013
[Mn]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Spring	BB and AR	0.000
		AR and CM	0.000
		AR and PF	0.000
		CM and PF	0.033
		Comparison:	
	BB	Autumn and Spring	0.000
		Winter and Spring	0.000
	AR	Summer and Spring	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.000
	CM	Summer and Spring	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.000
	PF	Autumn and Spring	0.000
[Zn]			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	AR and CM	0.041
	Autumn	AR and PF	0.034
	Winter	BB and AR	0.007
		Comparison:	
	BB	Autumn and Winter	0.024
[Cd]			
Stations	Tukey HSD	**Comparison:**	
		AR and CM	0.000
		AR and PF	0.004
[Pb]			
Seasons*Stations	Bonferroni	**Comparison:**	

Autumn	BB and AR	0.036
	AR and CM	0.005
	AR and PF	0.005
Winter	BB and CM	0.017
	Comparison:	
BB	Winter and Spring	0.034

Attachment 6 – Two-Way ANOVA and post-hoc tests results for biometrical parameters (L3 - ; body size and wet weight) considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ - value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
L3				
Seasons*Stations	6	0.514	3.795	0.001
Body Size				
Seasons*Stations	6	6814.064	24.983	0.000
Wet Weight				
Seasons*Stations	6	1099069.509	41.865	0.000

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
L3			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	BB and CM	0.000
		BB and PF	0.000
		AR and CM	0.031
		AR and PF	0.002
	Winter	BB and CM	0.000
		AR and CM	0.000
	Spring	BB and CM	0.000
		BB and PF	0.003
		AR and CM	0.000
		AR and PF	0.000
		Comparison:	
	BB	Autumn and Spring	0.000
		Winter and Spring	0.000
	AR	Summer and Spring	0.007
		Autumn and Spring	0.000
		Winter and Spring	0.000
	CM	Summer and Spring	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.000
	PF	Autumn and Spring	0.000
Body Size			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
		AR and PF	0.000
		CM and PF	0.001
	Winter	BB and CM	0.000
		AR and CM	0.000
	Spring	BB and CM	0.000
		BB and PF	0.031
		AR and CM	0.000
		AR and PF	0.000
		CM and PF	0.000
		Comparison:	
	BB	Autumn and Winter	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.043
	AR	Summer and Winter	0.022
	CM	Summer and Winter	0.000
		Summer and Spring	0.000
		Autumn and Winter	0.000
		Autumn and Spring	0.000

		Winter and Spring	0.005
Wet Weight			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	BB and AR	0.000
		BB and CM	0.000
		BB and PF	0.000
		AR and PF	0.000
		CM and PF	0.005
	Winter	BB and CM	0.000
		AR and CM	0.000
	Spring	BB and AR	0.012
		BB and CM	0.000
		BB and PF	0.021
		AR and CM	0.000
		AR and PF	0.000
		CM and PF	0.000
		Comparison:	
	BB	Autumn and Spring	0.000
		Winter and Spring	0.005
	AR	Autumn and Spring	0.006
	CM	Summer and Winter	0.000
		Summer and Spring	0.000
		Autumn and Winter	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.002

Attachment 7 – Two-Way ANOVA and post-hoc tests results for bioaccumulation factors (BAF) of *H. diversicolor* considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ -value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
$\mathrm{Cu_{BAF}}$				
Seasons*Stations	6	28.706	2.661	0.019
$\mathrm{Fe_{BAF}}$				
Stations	3	0.093	26.086	0.000
$\mathrm{Mn_{BAF}}$				
Seasons*Stations	6	2.271	2.96	0.010
$\mathrm{Zn_{BAF}}$				
Seasons*Stations	6	602.541	4.659	0.000
$\mathrm{Cd_{BAF}}$				
Seasons*Stations	6	1936455	3.847	0.002
$\mathrm{Pb_{BAF}}$				
Seasons*Stations	6	7.357	9.437	0.000

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
$\mathrm{Cu_{BAF}}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	AR and CM	0.000
		Comparison:	
	AR	Summer and Autumn	0.032
		Summer and Winter	0.031
		Summer and Spring	0.000
$\mathrm{Fe_{BAF}}$			
Stations	Tukey HSD	**Comparison:**	
		BB and CM	0.000
		AR and CM	0.000
		CM and PF	0.000
$\mathrm{Mn_{BAF}}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Spring	BB and CM	0.001
		BB and PF	0.000
		AR and CM	0.043
		AR and PF	0.001
		Comparison:	
	CM	Summer and Spring	0.002
	PF	Autumn and Spring	0.002
$\mathrm{Zn_{BAF}}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	AR and CM	0.044
	Winter	BB and AR	0.000
		AR and CM	0.000
	Spring	BB and AR	0.000
		BB and PF	0.000
		AR and CM	0.000
		CM and PF	0.000
		Comparison:	
	AR	Summer and Winter	0.000
		Summer and Spring	0.002
		Autumn and Winter	0.002
		Autumn and Spring	0.028
	PF	Autumn and Spring	0.000
$\mathrm{Cd_{BAF}}$			
Seasons*Stations	Bonferroni	**Comparison:**	

	Winter	BB and AR	0.000
		BB and CM	0.000
		Comparison:	
	BB	Autumn and Winter	0.000
		Winter and Spring	0.000
Pb$_{BAF}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	BB and AR	0.000
		AR and CM	0.000
		AR and PF	0.000
		Comparison:	
	AR	Summer and Autumn	0.000
		Autumn and Winter	0.000
		Autumn and Spring	0.000

Attachment 8 – Two-Way ANOVA and post-hoc tests results for concentration factors (CF) of *H. diversicolor* considering the effects of stations *Barrosa*'s Branch (BB), *Arnóia* and *Real* rivers (AR), *Covão dos Musaranhos* (CM) and *Poça das Ferrarias* (PF) and seasons (summer, autumn, winter and spring) as factors. Only significant results were represented (ρ -value < 0.05). df – degrees of freedom; MS – mean square.

TWOWAY ANOVA				
Source of variation	**df**	**MS**	**F-statistic**	**p-value**
Cu_{CF}				
Seasons*Stations	6	868493.335	20.275	0.000
Fe_{CF}				
Seasons*Stations	6	60967.483	4.485	0.000
Mn_{CF}				
Seasons*Stations	6	25504.863	24.960	0.000
Zn_{CF}				
Seasons*Stations	6	173099260.5	112.322	0.000
Cd_{CF}				
Seasons*Stations	6	576.322	6.268	0.000
Pb_{CF}				
Seasons*Stations	6	2.061	4.646	0.000

Post-hoc tests			
Source of variation	**Test**	**Condition**	**p-value**
Cu_{CF}			
Seasons*Stations	Bonferroni	**Comparison:**	
	Summer	AR and CM	0.000
		Comparison:	
	AR	Summer and Autumn	0.000
		Summer and Winter	0.000
		Summer and Spring	0.000
Fe_{CF}			
Seasons*Stations	Bonferroni	**Comparison:**	
	Winter	BB and CM	0.000
		AR and CM	0.000
	Spring	CM and PF	0.000
		Comparison:	
	BB	Winter and Spring	0.047
	CM	Summer and Winter	0.001
		Summer and Spring	0.037
		Autumn and Winter	0.000
		Autumn and Spring	0.012
Mn_{CF}			
Seasons*Stations	Bonferroni	**Comparison:**	
	Spring	BB and AR	0.000
		BB and CM	0.000
		AR and CM	0.000
		AR and PF	0.000
		CM and PF	0.000
		Comparison:	
	BB	Autumn and Spring	0.000
		Winter and Spring	0.000
	AR	Summer and Spring	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.000
	CM	Summer and Spring	0.000
		Autumn and Spring	0.000

		Winter and Spring	0.000
	PF	Autumn and Spring	0.000
Zn$_{CF}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Winter	BB and AR	0.000
		AR and CM	0.000
	Spring	BB and CM	0.000
		BB and PF	0.000
		AR and CM	0.000
		AR and PF	0.001
		CM and PF	0.000
		Comparison:	
	AR	Summer and Winter	0.000
		Autumn and Winter	0.000
		Winter and Spring	0.000
	CM	Summer and Spring	0.000
		Autumn and Spring	0.000
		Winter and Spring	0.000
	PF	Autumn and Spring	0.000
Cd$_{CF}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	BB and AR	0.000
		AR and CM	0.000
		AR and PF	0.000
	Winter	BB and AR	0.015
		AR and CM	0.016
		Comparison:	
	AR	Summer and Autumn	0.000
		Autumn and Winter	0.001
		Autumn and Spring	0.000
		Winter and Spring	0.040
Pb$_{CF}$			
Seasons*Stations	Bonferroni	**Comparison:**	
	Autumn	BB and AR	0.036
		BB and CM	0.039
		BB and PF	0.021
		AR and CM	0.000
		AR and PF	0.000
		Comparison:	
	BB	Autumn and Spring	0.010
	AR	Summer and Autumn	0.000
		Autumn and Winter	0.000
		Autumn and Spring	0.000