Universidade de Aveiro
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Evaluation of seasonality and elements contamination in the performance of the polychaete *Diopatra neapolitana* from Ria de Aveiro

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Adília da Conceição Marques de Oliveira Pires, Investigadora Auxiliar do Departamento de Biologia & Centro de Estudos do Ambiente e Mar, Universidade de Aveiro, e coorientação do Doutor Paulo Jorge da Rocha Cardoso, Investigador Auxiliar do Departamento de Biologia & Centro de Estudos do Ambiente e Mar, Universidade de Aveiro.

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Aos meus pais e à minha irmã.
Consegui!

o júri

Presidente	Doutora Isabel Maria Cunha Antunes Lopes Investigadora Principal em Regime Laboral, Universidade de Aveiro
Arguente	Doutora Cátia Alexandra Ribeiro Venâncio Investigadora Auxiliar, Universidade de Coimbra
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palavras-chave Sazonalidade; bioacumulação de elementos; *Diopatra neapolitana*; marcadores bioquímicos

resumo

Os estuários são ecossistemas de interface que unem os ambientes continental e marinho, recebendo contribuições biogeoquímicas ativas da costa, rios e mares costeiros. Como os estuários são uma mistura de água doce e salgada, eles atuam como zonas de descarga e fontes de poluentes. A Ria de Aveiro é um bom exemplo de sistema costeiro ecologicamente ativo que ao longo dos anos tem sofrido mais com atividades antropogénicas, como a agricultura e a descarga de efluentes da indústria e também porque contém uma grande variedade de espécies que ali habitam.

Os metais são elementos que existem na natureza por meio natural, porém, devido a atividades antropogénicas, estes elementos podem contaminar as águas, devido a descargas ou à alteração da geoquímica destes. Este fenómeno tem impactos negativos na vida marinha acabando por se tornarem prejudiciais nos níveis superiores da cadeia trófica.

Os poliquetas são normalmente o grupo mais abundante em sistemas estuarinos e são uma importante fonte de alimento para espécies de vários níveis tróficos. Algumas espécies também têm importância económica. Muitos estudos já foram feitos com estes organismos sobre impactos das alterações climáticas e contaminantes das águas, sendo por isso também conhecidas como organismos sentinela. *Diopatra neapolitana* é uma espécie com grande relevância ecológica na Ria de Aveiro e é muito utilizada para os estudos mencionados anteriormente.

Este trabalho tem como principal objetivo compreender como é que a sazonalidade tem impacto na bioacumulação de metais no poliqueta *D. neapolitana* e determinar de que forma é que esta espécie responde a este tipo de contaminantes, através de marcadores bioquímicos. Para isso, foram colhidos organismos de *D. neapolitana* ao longo das quatro estações do ano, em 2018 e 2019 em cinco locais diferentes da Ria de Aveiro.

De forma geral, os resultados obtidos neste trabalho demonstraram que o outono foi a estação em que se observou uma maior concentração de elementos nos sedimentos, principalmente no local Cale do Ouro. No entanto, em relação às amostras de tecido, o outono e o verão foram as estações do ano em que os organismos acumularam mais elementos. Esses resultados também indicam que os fatores abióticos como a temperatura, salinidade, pH, oxigénio dissolvido, conteúdo em finos e matéria orgânica influenciam a disponibilidade dos elementos. Além disso, os elementos acumularam de forma diferente entre as frações nos tecidos do poliqueta, tendo-se observado uma maior bioacumulação de elementos na fração insolúvel. Em relação às defesas antioxidantes, foi observada maior atividade enzimática durante a primavera e o verão, principalmente devido às altas temperaturas e à bioacumulação de elementos. Os parâmetros relacionados com o metabolismo apresentaram níveis mais elevados na primavera e no outono também principalmente devido à bioacumulação de elementos. O dano lipídico foi maior durante o inverno e isso ocorreu principalmente devido às características físicas e químicas dos locais de amostragem, como a diminuição da salinidade e da temperatura.

keywords

Seasonality; elements bioaccumulation; *Diopatra neapolitana*; biochemical markers

abstract

Estuaries are interface ecosystems that unite the continental and marine environments, receiving active biogeochemical contributions from the coast, rivers and coastal seas. As estuaries are a mixture of fresh and saltwater, they act as discharge zones and sources of pollutants. Ria de Aveiro is a good example of an ecologically active coastal system that over the years has suffered more from anthropogenic activities, such as agriculture and effluent discharge. Ria de Aveiro also contains a wide variety of species that live there.

Metals are elements that naturally exist in nature, however, due to anthropogenic activities, these elements can contaminate the waters, due to discharges or alteration of their geochemistry. This phenomenon has negative impacts on marine life and becomes harmful at the food chain's upper levels.

Polychaetes are usually the most abundant group in estuarine systems and are an important food source for species of various trophic levels. Some species are also of economic importance. Several studies have already been done with these organisms on the impacts of climate change and water contaminants, being known as sentinel organisms. *Diopatra neapolitana* is a species with great ecological relevance in the Ria de Aveiro and is widely used for the studies mentioned above, that is why we decided to use this organism to this study.

This work has the main objective to understand how the seasonality impacts the bioaccumulation of elements in the polychaete *D. neapolitana* and determine how it responds to this type of contaminants through biochemical markers. For this, *D. neapolitana* organisms were harvested over the four seasons of the year, in 2018 and 2019 in five different locations of Ria de Aveiro.

In general, the results obtained in this work demonstrated that Autumn was the season in which the sediments presented higher concentration of elements, mainly in the site Cale do Ouro. However, concerning tissue samples, Autumn and Summer were the seasons when organisms bioaccumulated more elements. These results also indicate that abiotic factors like temperature, salinity, pH, dissolved oxygen, fines content and organic matter influence the availability of the elements. Also, elements bioaccumulated differently in polychaete tissues fractions since insoluble fraction accumulated more elements than soluble fraction. Regarding antioxidant defenses, higher enzymatic activity was observed during Spring and Summer, mainly due to high temperatures and the bioaccumulation of elements. The metabolism-related parameters showed higher levels during Spring and Autumn also mainly due to elements bioaccumulation. Lipid damage was higher during the Winter, and this was mainly due to the physical and chemical characteristics of the sampling sites, such as salinity and temperature decrease.

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Abbreviations

Al- Aluminum

As- Arsenic

Ba- Barium

Be- Beryllium

BSA- Bovine Serum Albumin

BSS- Balanced Salt Solution

Ca- Calcium

CAT- Catalase

Cd- Cadmium

CD- Cale do Ouro

CDNB- 1-Chloro-2,4-Dinitrobenzene

CN- Costa Nova

Co- Cobalt

Cr- Chromium

Cu- Copper

DNPH- 2,4-Dinitrophenylhydrazine

DNTB- 5,5-dithio-bis-(2-nitrobenzoic acid)

DO- Dissolved Oxygen

DTT- Dithiothreitol

DW- Dry Weight

EDTA- Ethylenediamine Tetra acetic Acid

Eh- Redox Potential

ETS- Electron Transport System

Fe- Iron

FW- Fresh Weight

GLY- Glycogen

GSH- Glutathione

GSTs- Glutathione S-Transferases

ICP-MS- Inductively Coupled Plasma-Mass Spectrometry

K- Potassium

Li- Lithium

LPO- Lipid Peroxidation

M- Murtosa

MDA- Malondialdehyde

Mg- Magnesium

$MgSO_4$- Magnesium Sulphate

Mn- Manganese

NBT- Nitro Blue Tetrazolium Salt

Ni- Nickel

NPT- Non-Protein Thiols

P- Phosphorus

Pb- Lead

PCO- Principal Coordinate Analysis

ProC- Protein Carbonylation

PROT- Protein

PVP- Polyvinylpyrrolidone

ROS- Reactive Oxygen Species

S- Sulfur

Sb- Antimony

SJ- São Jacinto

Sn- Tin

SOD- Superoxide Dismutase

T- Torreira

TBA- 2-Thiobartbituric Acid

TCA- Trichloroacetic Acid

Tl- Thallium

V- Vanadium

XO- Xanthine Oxidase

W- Tungsten

Zn- Zinc

Chapter I- General introduction

General introduction

1. Estuaries and environmental alterations: Ria de Aveiro

An estuary is a semi-closed body of water connected to the sea, up to the limit of the tide or the limit of salt intrusion and receiving runoff from freshwater, recognizing that the influx of fresh water may not be perennial and that the connection to the sea may be closed for part of the year and that the influence of the tide can be negligible [1]. Estuaries are also interface ecosystems that unite continental and marine environments, receiving active biogeochemical contributions from the coast, rivers and coastal seas [2]. They are of great importance in physical oceanography, marine geology, marine ecology, marine biology, hydraulic engineering, shipping, and pollution monitoring and assessment [3].

Since estuaries are a mixture of freshwater and saline water, they act as sinks and source of pollutants. This depends on the geographical source of the contaminants (marine, fluvial, internal and atmospheric), their biological and chemical nature and with temporal variations in tidal amplitude, river flow, seasons, winds and waves [4]. In fact, the estuarine waters constitute naturally stressed, highly variable ecosystems that are also exposed to high levels of anthropogenic stress making these environments the hardest to endure for inhabiting organisms [5], [6].

Despite these challenges, estuaries provide habitats and food source to numerous organisms within the intertidal and subtidal zones, making these ecosystems extremely important in ecological and economical terms. Organisms that live in these environments are subjected to various climate alterations such as temperature, salinity and oxygen availability, for example [7], [8]. These alterations although can happen due to natural causes but might also be related to the discharge of effluents resulting from anthropogenic activity, like domestic and agriculture activities, that alter the pH, temperature and salinity of the waters endangering the ecosystem [9], [10]. Sediments can also undergo geochemical changes due to changes in the chemical parameters of the water, which can result in negative impacts on the organisms that inhabits there [11].

Ria de Aveiro is in the northeast coast of Portugal (40º38’N, 8º45’W) constituting a permanent habitat to various species of fauna and flora. There are many activities in Ria de Aveiro that goes from economical (e.g. salt production, aquaculture, fisheries, agriculture) to touristic activities [12]. In its 45 km of extension, from Ovar to Mira, and 10 km of maximum width, the Ria covers an area of 83 km^2 in high tide (spring tide), which reduces to 66 km^2 in low tide [13]. This system is linked to the Atlantic Ocean through an artificial channel of 1.3 km of length, 350 m of width and 20 m of depth having a maximum width of 10 km and 45 km of length. In Spring, the area is 83 km^2 at high tide, being reduced to 66 km^2 at low tide. The average depth of the lagoon is approximately 1 m, but the channel located near the entrance to the Ria is about 20 m [14]. Ria de Aveiro is considered an mesotidal lagoon [15] and it extends into four main channels (Mira, São Jacinto, Ílhavo and Espinheiro), which have several branches and interconnections (with the exception of the Mira channel) and contribute to the complexity of the system [16]. The lagoon receives freshwater from two main rivers Antuã and Vouga and two other small rivers, Boco and Caster, contributes with a lower flow [2], [17]–[19] .

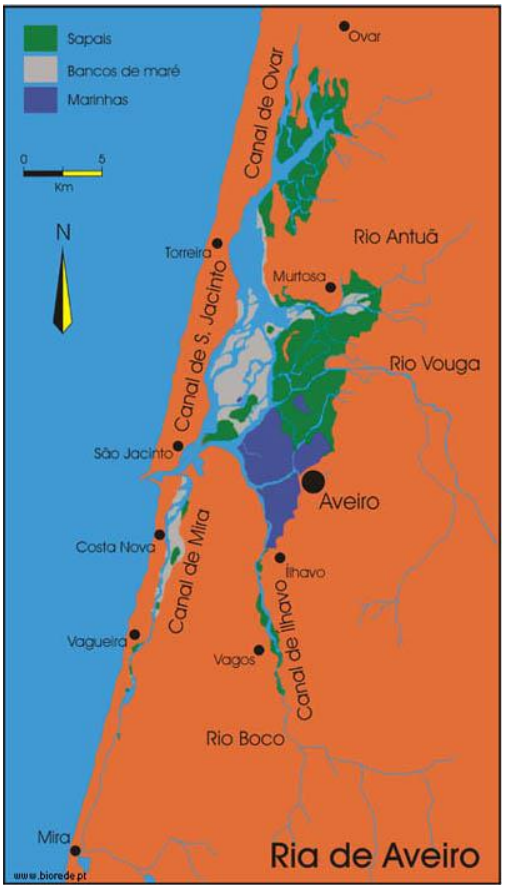


Figure 1. 1 Map of Ria de Aveiro from Biorede [19].

Ria de Aveiro is a good example of an ecologically active coastal system that over the years has been suffering more with anthropogenic activities, like agriculture and discharge of effluents containing various toxic elements (e.g. polycyclic aromatic hydrocarbons, pharmaceuticals and metals) which has contributed to the degradation of the ecosystem and water quality [2], [20]–[22]. However, in the last years, measures have been adopted to reduce the effluent load to the Ria [12].

Since the Ria de Aveiro is permanently linked to the sea, it contains a wide range of species that inhabit there such as phytoplankton, zooplankton, bivalves, crustaceans, fish, birds and polychaetes [9], [12], [23]–[25]. Therefore, is important to monitor the consequences that these anthropogenic activities have in the organisms that habit this ecosystem.

2. Seasonality and spatial variation

Physical and chemical parameters of the water (e.g. temperature, salinity, dissolved oxygen, pH and organic matter) are important features for the survival and well-being of organisms [8], however, these characteristics varies among the seasons and sites. In a monitoring program, it is very important to study several sites with different characteristics to evaluate how do organisms behave in those sites under those conditions, however, is also important to study how the same organism from different sites reacts to seasonality changes. Despite these changes, aquatic organisms are able to adapt up to a certain tolerance limit which depends on the natural environmental conditions for which each organism is acclimatized [26], [27].

Studies concerning biota facilitate the detection of negative consequences of chemical exposure before becoming significant in conservation or ecological impacts [28]. Approaches of ecosystem biomonitoring have been based in the effects in benthic organisms, by the assessment of alterations at the community level (benthic community parameters), and more recently, on individual and cellular levels (physiological and biochemical markers), not only to assess the impacts of pollutants but also to investigate alterations derived from climate change, namely when related to weather events [29].

Generally, studies that measure the impact of pollutants in community levels or using organisms' biochemical responses are made only at a specific time of the year. However, although they are essential and give relevant information, this type of studies have some limitations: i) usually fail to detect subtle effects masked by biotic and abiotic impacts, such as habitat degradation [30]; ii) there is a main advantage of anticipating the effects before they reach higher levels of biological organization [31]. To overcome these factors that might vary among seasons is important to make studies with longer duration to eliminate these variations that can compromise the interpretation of biomonitoring data.

However, not only organisms' biochemical responses give us important data to understand what variations occurs along the seasons and sites. Pollutants also accumulate in sediments and their bioavailability is related to the physical and chemical features of the sites and how these features change along the seasons and to the presence of the fauna existing in that environment [32].

3. Element contamination

Elements are natural constituents of the environment and vary in concentrations across the regions, however, over the last decades, elements concentrations have increased in coastal and estuarine ecosystems due to human activities [33]. While organic pollutants may be degraded into less harmful components through biological or chemical processes, elements are not considered degradable components because they can accumulate on sediments or be released by sediments, acting like a return source to overlying water via natural or anthropogenic disturbance [11]. Tidal currents and bioturbation (burrowing and irrigation activities of benthic organisms) in estuarine sediments increase the depth of oxygen penetration and create microenvironments where both oxidized and reduced forms of iron (Fe), manganese (Mn), and sulfur (S) coexist in a dynamic equilibrium under subtoxic conditions [34]–[36].

Sediments are a major reservoir of persistent contaminants storage in aquatic environments, therefore, they become a potential source to interstitial waters and inhabiting organisms, namely benthic species [37], [38]. Benthic macroinvertebrate species are in close contact with sediment for long periods and can accumulate large amounts of elements, giving information about the environmental contamination and offering the possibility of use as bioindicators of metal pollution in coastal areas (e.g. [39], [40]). The effects of pollution by elements on the environment and local organisms can be substantial or long lasting, moreover, these elements, like metal(loid)s can enter the food chain and could have deleterious effects on the human being [41], [42]. However, not all elements enter in the food chain and are toxic to organisms [43], but, is important to monitor these phenomenon since bioaccumulation in the organisms is a critical factor when it comes to evaluate the adverse effects of pollutants in ecosystems [44].

4. Polychaetes as bioindicators: *Diopatra neapolitana*

Polychaetes are the group that comprises most of the diversity of annelids and can be found mainly in all kinds of the marine environment from the tidal zone to zones with greater depth [45], [46]. Usually are the most abundant annelid group in marine ecosystems, becoming key elements on estuarine and coastal food webs [47]. This makes them the major food source for many fishes and crustaceans, making them important organisms to transfer contaminants to higher trophic levels [48], [49]. Additionally, many species of polychaetes are economically important because they are used as fish bait and as food in fish and bivalve aquacultures [50], [51].

These organisms predominantly live buried in sediment, being able to build tubes, holes or even galleries (e.g. *Diopatra neapolitana*, Delle Chiaje, 1841; *Arenicola marina*, Linnaeus, 1758; *Glycera dibranchiata*, Ehlers, 1868) but also can live in the water column. Also, as they inhabit most in the sediment, they tend to accumulate more contaminants because their feeding strategy involves ingesting sediments, leading to maximum exposure to contaminants by sediment and water [52]. Growth, reproduction, feeding and survival have been important points used to determine individual responses, and these endpoints can be correlated with exposure to pollutants [53]. Many authors have already done studies to try to understand the impacts caused in polychaetes due to the accumulation of pollutants, for example using *Hediste diversicolor* [54], *Diopatra neapolitana* [55], *Arenicola marina* [56], *Marphisa sanguinea* [57], among others. Therefore, polychaetes are known as sentinel organisms because they are able to survive sudden variations in the environment in which they live and are good indicators of the level of pollution.

In this study, the polychaete *Diopatra neapolitana* (Delle Chiaje, 1841) was used because this species is usually available all year round and frequently occurs in high densities [50], [58]–[61]. This tubiculous onuphid is common in Ria de Aveiro inhabiting intertidal sandflats and shallow subtidal areas, growing up to 70 cm [25]. This species has been reported to be found in various places of the world, such as Red Sea, Indian Ocean, Mediterranean Sea and Atlantic Ocean [59], [62]–[64].

Figure 1. 2 Diopatra neapolitana's tube in Ria de Aveiro, from Biorede [61].

This species plays an ecologically important role because its tubes stabilize the sediment, increasing the structural complexity and, consequently, its biodiversity, providing refuge and protecting from predation, and facilitating the settlement of some algae species [65], [66]. It is intensively exploited as fish bait in coastal lagoons, constituting an important source of income and has a wide distribution in the intertidal and subtidal zones, including the Ria de Aveiro [50], [58], [59]. Some studies demonstrated that this species is a good bioindicator of environmental changes [67], [68], organic matter enrichment [69], metals accumulation [55], chemical compounds [56] and nanomaterials [70]. Their reproductive period occurs especially from june to august [71].

5. Biochemical parameters

Environmental monitoring is important to understand the changes that are occurring and if these changes affects the biologic systems [72]. Aquatic systems are in constant contact with contaminants and this contact can have negative effects in the future. There are many procedures to detect if there is any impact of anthropogenic contamination, for example chemical and geochemical analysis; biochemical, physiological, histopathological and behavioral biomarkers that demonstrate that there is exposure to something and in some cases, show adverse effects to contaminants [73]. Biomarkers have been used to environmental hazard assessment, hazard assessment in populations of endangered species and identification of species at risk since biomarkers represent rapid responses to toxicant exposure, providing an early warning signal of possible long-term effects [74]. Therefore, it is possible to say that a biomarker is a biological response to chemical or chemicals that give a measure to exposure and sometimes, a toxic effect [72]. Biomarkers can be used in three approaches: the first one is by passive bio monitorization (as in this study), the second one is by active bio monitorization, where the sentinel species are transported to a specific local of study, and the third one is in laboratory studies, where the species are under stressful conditions and the main objective is to access the consequences [14].

Not all biomarkers respond in the same way. Some biomarkers respond to general environmental stress, and other biomarkers respond to specific groups of compounds [75], [76]. Therefore, it is essential to use multiple biomarkers to get a more accurate answer about the site's contamination level and how it affects those organisms under study.

Reactive oxygen species are the principal class of radicals species generated in living systems and can cause effects denominated as oxidative stress [77]. This oxidative stress is increasingly used as a biomarker of aquatic contamination [78]–[80]. It is important to perform biomarker measurements in invertebrates since it represents 95% of all animal species; these populations are often numerous, therefore, it is possible to analyze without putting populations at risk, they are found occupying all trophic levels, and the legislation for the use of invertebrates is reduced [72], [81].

In this work, we used biomarkers related to metabolism (protein, glycogen and electron transport system), antioxidant defenses (catalase, superoxide dismutase, glutathione S-transferases and non-protein thiols), and oxidative damage (lipid peroxidation and protein carbonylation).

6. Objective

The present study aimed to characterize how seasonality and sediment characteristics influence element concentration in sediments and assess the impact of elements bioaccumulation and cellular partitioning in the polychaete *D. neapolitana*. Moreover, this work also aims to understand the effect of abiotic factors and elements bioaccumulation on this species, through several biochemical parameters, such as energy metabolism, oxidative stress, cell membranes, and proteins damage.

The present dissertation was divided into four chapters. Chapter I is the current general introduction. Chapter II addresses the concentration of major and trace elements in sediment samples and in the tissue of *D. neapolitana* and evaluate if these concentrations varied among seasons and sites. Chapter III will be presented the biochemical analysis in the polychaete *Diopatra neapolitana* collected in the sampling sites to understand the impact of elements accumulation among seasons and different sites. Chapter IV comprises the final considerations and future perspectives.

Chapter II- Major and trace elements and physical and chemical characteristics of sediments

Abstract

Coastal systems often serve as sinks for toxic elements, and seasonality has been responsible for many changes in the physical and chemical parameters of waters and sediments, leading to geochemical alterations in aquatic systems. The effects of these changes may also alter elements uptake rates. These fluctuations are expected to lead to important changes in estuarine communities, mainly on those organisms living in direct contact with sediments, such as polychaetes. Ria de Aveiro is a coastal zone with many branches that lead to many water mixtures, containing contaminants from anthropogenic activity that once entering in marine ecosystems will become dangerous to marine organisms that inhabit these environments. Thus, this study aimed to assess how elements concentrations varied along seasons in several sites from Ria de Aveiro lagoon. Moreover, the polychaete *Diopatra neapolitana* was also harvested to evaluate elements bioaccumulation among seasons and sites. Results showed that sediments from site Cale do Ouro in Autumn season had higher major and trace elements concentration and the site Torreira in Winter season had less abundant elements. High bioaccumulation of the major elements Na and K were found in the soluble fraction and a higher bioaccumulation of Ca, Fe, Mg and P in the insoluble fraction of polychaetes. Regarding trace elements, they were accumulated differently among fractions: while insoluble fraction bioaccumulated more trace elements, like As, Ba, Cd, Co, Li, Mn, Sb, V and Zn while soluble fraction had higher levels of the elements As, Cu, Pb, Sb and Sn.

Keywords: Major and trace elements, contamination, Polychaete, physical and chemical parameters, seasonality.

Introduction

Over time, extreme events have affected the sea level and temperature, leading to other physical and chemical alterations in water parameters affecting marine populations [58]. Therefore, it became more common to study the impacts that these alterations have on marine organisms (e.g [82]–[85]). These studies generally have the purpose of understanding which impacts these alterations have in those organisms on physical and biochemical pathways and if these alterations are only related to these parameters or if pollutants also play a role in it (e.g [67], [86]). These pollutants can be from anthropogenic origin, like drugs or toxic discharges, which leads to water contamination and can accumulate in sediments, affecting the organisms. However, some elements occur in the environment naturally, but they can suffer geochemical alterations and become toxic due to abiotic alterations, as temperature increase or salinity shifts [11].

Element contamination has also become an emerging theme throughout the years. Therefore, its studies also have increased to understand what impacts these elements had in marine organisms (e.g [16], [83], [87]). Many marine species from lower trophic levels, as polychaetes, live burrowed in sediment, being in intimate contact with elements, present in sediment and in pore water, that could be accumulated by them and consequently enter in the food chain reaching higher trophic levels and contaminating all food web [88]. Other authors have already seen elements bioaccumulation in *Diopatra neapolitana* (e.g. [55], [89]), *Hediste diversicolor, Neanthes succinea* and *Perinereis cultrifera* (e.g [90], [91]).

Previous studies stated that environmental changes, as salinity and temperature, affect chemical speciation and elements solubility, changing their dissolved concentrations in aquatic systems, with consequences on their bioavailability (e.g [92], [93]). Moreover, these abiotic alterations are known to impact polychaetes, with implications to population sustainability [67], [68], [94]. Altogether, these changes may impact coastal systems biodiversity (e.g. [95]).

Among the most used bioindicator polychaete species, *Diopatra neapolitana* presents a wide geographical distribution, [50], [58], [59]. This species has been shown to be a good bioindicator of inorganic and organic contamination [69], [96]–[98] and abiotic factors, as temperature shifts, salinity changes and water acidification [67], [94], [99].

Although several studies reported that abiotic factors affect marine organisms, less is known on the impacts of these environmental changes on elements availability. Information integrating both ecosystem components is still lacking, making difficult to predict the effects of environmental changes on marine systems. Although challenging, assessing abiotic factors alterations that occur along the year in marine ecosystems contaminated with elements and the impact on marine organisms is extremely important, especially on those living in direct contact with sediments, as polychaetes.

Thus, this study aims to assess the bioaccumulation of major and trace elements in the polychaete *D. neapolitana* collected at several sites along Ria de Aveiro lagoon and evaluate how it varies along the four seasons of the year (Autumn, Winter, Spring and Summer) to understand the impact of abiotic factors on elements bioaccumulation.

Methodology

1. Study area and field sampling

During Autumn of 2018, Winter, Spring and Summer of 2019, sediments were collected from five different sites (Cale do Ouro - CD; Murtosa - M; São Jacinto - SJ; Torreira – T; Costa Nova- CN) of Ria de Aveiro. The Ria of Aveiro is a shallow coastal lagoon located on the Atlantic coast of northwest Portugal (40'38'N, 8'44'W), separated from the sea by a sand bar, and characterized by narrow channels and the existence of significant intertidal areas, namely mudflats and salt marshes [16].

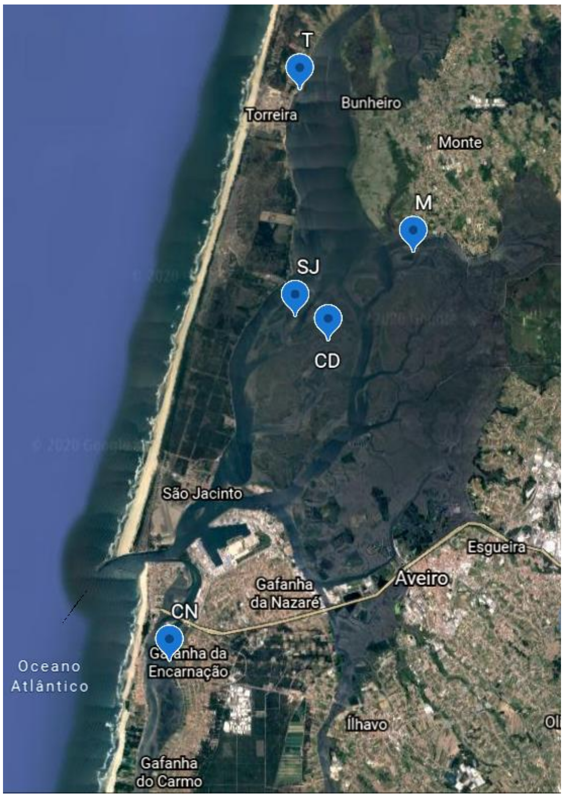


Figure 2. 1 Map of the sampling site from Google Earth. CD: Cale do Ouro; M: Murtosa; SJ: São Jacinto; T: Torreira; CN: Costa Nova.

In situ measurements of pH, redox potential (Eh), dissolved oxygen (DO), salinity and temperature were measured in the sediment-water interface using a handheld multiparametric waterproof meter- Model HI98194 Hanna Instruments at each sampling area and season (Table 2.1). Sediments from each area were also collected and transported to the laboratory and used for grain size analysis (% fines), organic matter (% OM) content determination and for total elements quantification.

2. Sediment samples

The concentrations of aluminum (Al), arsenic (As), barium (Ba), beryllium (Be), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), lithium (Li), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), antimony (Sb), tin (Sn), thallium (Tl), vanadium (V), tungsten (W) and zinc (Zn) were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7700x) after acid digestion. A 250 mg of air-dried and homogenized sediment was added 1.5 mL of HCl and 4.5 mL of HNO_3 in Teflon vessels. After 24 h, the Teflon vessels were placed on a heating plate at 115 ºC and after 6 h the contents were transferred to a falcon tube. After adding 45 mL of ultrapure water, tubes were centrifuged and then read. A rigorous quality control was performed during these analyses, which included the analysis of blanks, duplicate samples, and certified reference materials. The precision and bias error of the chemical analysis was less than 10%.

Percentage of fines was determined following Quintino et al. (1989) [100]. The total organic matter content was determined by weight loss on ignition at 450 °C, during 5 h (Byers et al., 1978) [101] of 1 g sediment sample after an initial drying at 60 °C for 24 h.

3. Tissue samples

The concentrations of aluminum (Al), arsenic (As), barium (Ba), beryllium (Be), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), lithium (Li), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), antimony (Sb), tin (Sn), thallium (Tl), vanadium (V), tungsten (W) and zinc (Zn) were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7700x) after acid digestion. A 500 mg of homogenized tissue was subjected to subcellular fractionation by centrifugation at 1450 g, for 15 min at 4ºC. Fractionation resulted in the isolation of two distinct fractions: insoluble and soluble. According to Wallace et al, (2003) [102], the insoluble fraction can be defined as the unavailable element's concentration, precipitated in insoluble metal-rich granules, and cellular debris, while the soluble fraction can be defined as the element's concentration in its free form or bound to proteins present in the cytosol. To each fraction was added 1.5 mL of HCl and 4.5 mL of HNO_3 in Teflon vessels. After 24 h, the Teflon vessels were placed on a heating plate at 115 ºC and after 6 h the contents were transferred to a falcon tube. After adding 45 mL of ultrapure water, tubes were centrifuged and then read. A rigorous quality control was performed during these analyses, which included the analysis of blanks, duplicate samples, and certified reference materials. The precision and bias error of the chemical analysis was less than 10%.

4. Statistical analysis

Data from the physical and chemical parameters (pH, salinity, temperature, DO, Eh, % fines and % OM) and elements, per area and per season, were transformed (square root), normalized and used to calculate a Euclidean matrix between sampling areas using the PERMANOVA+ add-on in PRIMER v6 + [103]. A Principal Coordinates Ordination analysis (PCO) was used to visualize differences among areas and seasons. The environmental parameters highly correlated (r> 0.7) were represented as superimposed vectors in the graph.

Afterwards, the matrix containing data from the physical and chemical parameters and concentration of elements in sediments, bioaccumulation in *D. neapolitana* soluble and insoluble fractions of elements per sampling area and season was used to perform another PCO analysis. In the PCO graph, the variables presenting a correlation higher than 70 % with samples ordination were represented as superimposed vectors.

Also, data from element concentration in sediments and element accumulation in tissues were transformed (logarithmic transformation) using the program Metaboanalyst. This data was represented in heatmaps.

Results

1. Physical and chemical characteristics

Overall, Summer had higher salinity levels in all sites and SJ, T and CN the lowest levels during Autumn. The lowest DO level was during Spring in site M and the highest was in Spring in site CN. Eh varied between 67.27 mV and 255.57 mV with SJ during Summer presenting the lowest value and site T during Spring had the highest. Lowest levels of temperature were recorded during Winter and pH levels did not vary significantly between seasons and sites. Highest values of % OM were observed during Autumn in CD and Summer in CN. Lowest values were recorded during Spring and Summer in SJ. Regarding % fines, these values varied significantly among sites and seasons, being the highest value recorded in Autumn in CD and CN in Summer and the lowest values in Autumn at CN (Table

2.1). Site M during Winter does not have any values because it was not possible to collect samples due to climacteric and logistic conditions.

Table 1. 1. Physical and chemical characteristics of the sampling areas.

Area	Season	Salinity	Dissolved oxygen (mg/L)	Redox potential (mV)	pH	Temperature (°C)	% Organic matter	% Fines
Cale do Ouro (40.7007920°, -008.6859120°)	Autumn	36.39	6.72	86.23	8	16.4	6.30	53.20
	Winter	34.25	8.91	135.27	7.99	13.4	1.87	12.33
	Spring	37.43	7.11	83.87	7.93	18.37	1.90	7.09
	Summer	38.97	8.73	88.37	8.15	22.7	3.03	17.94
Murtosa (40.7198333° -008.6470167°)	Autumn	38.02	7.15	106.43	7.92	18.67	2.41	35.16
	Winter	-	-	-	-	-	-	-
	Spring	36.04	5.204	98.98	7.65	20.37	3.61	9.15
	Summer	38.73	6.63	147.57	7.93	21.9	2.28	10.48
São Jacinto (40.7066850°, -008.6973950°)	Autumn	33.87	8.56	143.67	7.99	17.43	1.64	44.77
	Winter	29.21	6.7	110.37	7.89	12.23	2.79	33.85
	Spring	35.26	6.03	205.07	7.81	20.9	0.68	5.50
	Summer	37.58	7.16	67.27	8.27	23.7	0.77	4.41
Torreira (40.7627500°, -008.6982667°)	Autumn	34.58	8.73	139.23	7.8	16.53	1.51	20.46
	Winter	23.78	5.91	101.87	7.77	10.97	2.44	29.31
	Spring	36.92	5.95	255.57	7.8	19.37	2.07	27.82
	Summer	37.74	7.1	130.27	8	22.3	1.84	5.89
Costa Nova (40.6118056°, -008.7433056°)	Autumn	34.68	6.9	189.8	7.92	16.53	1.34	4.35
	Winter	19.79	7.94	186.5	8.12	13.13	1.26	13.04
	Spring	36.29	9.75	113.26	8.22	20.17	1.52	24.24
	Summer	38.97	6.72	86.23	8	22.83	6.30	53.20

2. Major elements

2.1 Sediments

In Autumn, site CD showed a higher concentration of all major elements, except for Ca, in sediments. Overall, in Autumn was observed a higher concentration of major elements for all sites and in Spring, a lower concentration of these elements was observed (Figure 2.2). Major elements concentration found in sediments for each site and season are presented in Figure S2.1.

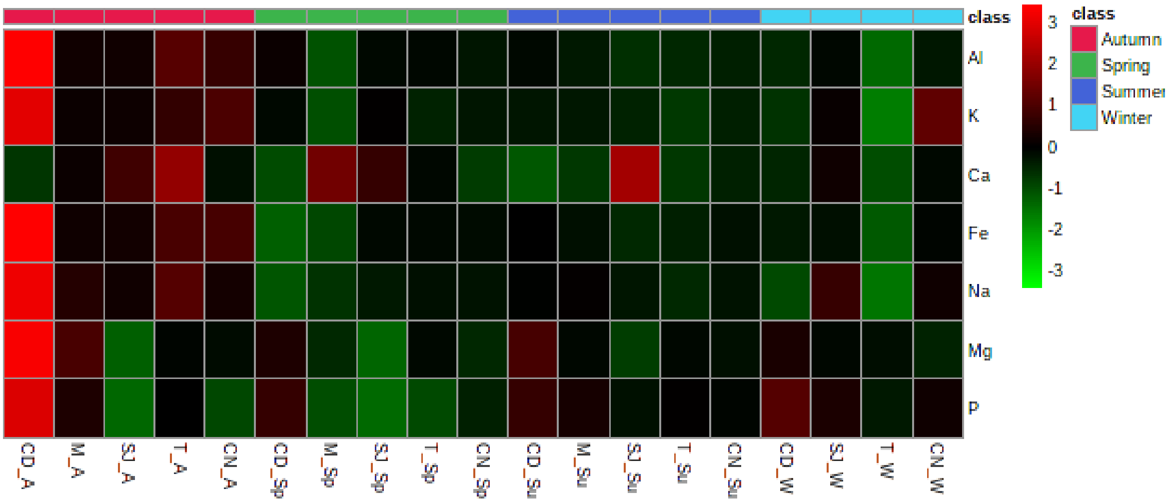


Figure 2. 2 Heatmap comparing major elements concentration (Al, Ca, Fe, K, Mg, Na and P) in sediments of sampling sites. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer**.**

Principal Coordinates Ordination analysis (PCO) regarding major elements concentration in sediments and physical-chemical characteristics of sediments and water of the sites where organisms were collected evidenced that together PCO1 and PCO2 explained 61% of the total variation obtained among areas (Figure 2.3). The results obtained revealed that samples were separated in three main groups. PCO1 explained 42% of total variation separating samples collected in Autumn (except site SJ), and samples from site SJ and site CN of Winter, site CD and site CN of Spring and Summer. The major elements Al, Fe, K, Mg, Na and P and physical and chemical parameters DO, % fines and % OM presented a positive correlation with axis 1 (r> 0.7), indicating that higher percentage of these characteristics are responsible by the higher concentrations of these major elements in the sediments (Figure 2.3). Axis 2 explained 19% of total variation separating Autumn, site M, site SJ and site T of Spring and Summer in the positive side. The major elements Al, Ca, Fe, K and Na present in the sediments had a positive correlation with this axis (r> 0.7). Winter and site CD and site CN of Spring and Summer were separated in the negative side of axis 2, and the major elements Mg and P and % fines, % OM, DO and pH were negative correlated with this axis (r> 0.7) (Figure 2.3).

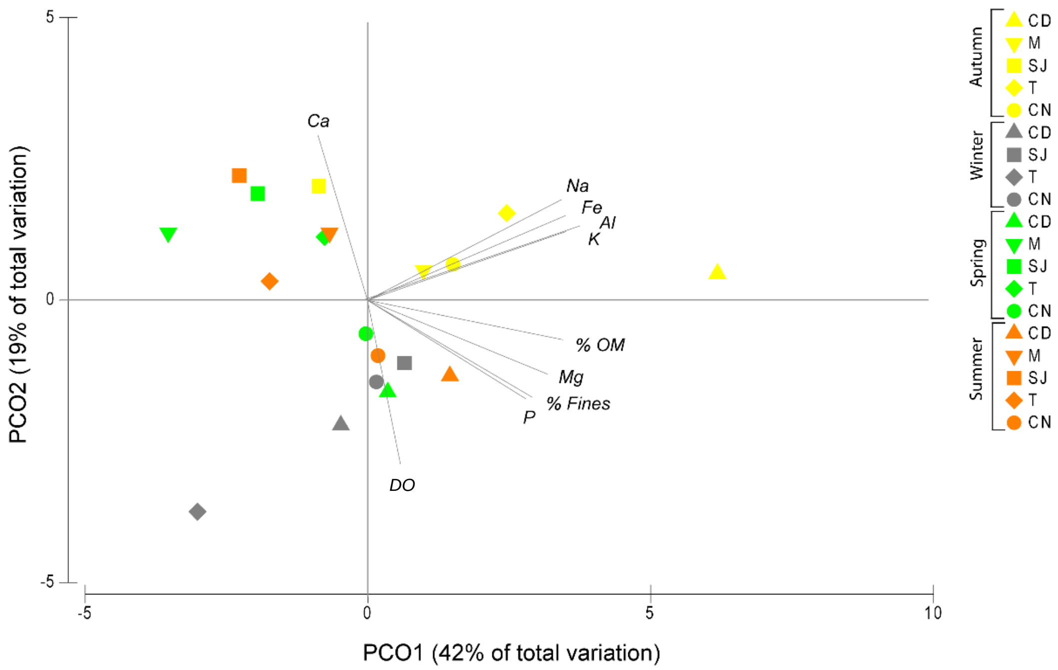


Figure 2. 3 Principal coordinate ordination analysis (PCO) of the sediments collected in sites and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely major elements concentration data and physical and chemical parameters (r> 0.7): Al; Ca; Fe; K; Mg; P; DO; pH; % Fines; % OM. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

2.2 Tissue samples

Regarding the bioaccumulation of major elements in tissue samples of the polychaetes, in the heatmap of Figure 2.4 it is possible to observe a separation between the concentration of these elements in soluble and insoluble fraction. Major elements like Al, Ca, Fe, Mg and P were higher in the insoluble fraction while Na and K were in higher concentrations in the tissues' soluble fraction. Major elements bioaccumulation detected in polychaetes tissue for each site and season are presented in Figure S2.2 and Figure S2.3. There were no significant differences of major elements bioaccumulation between seasons.

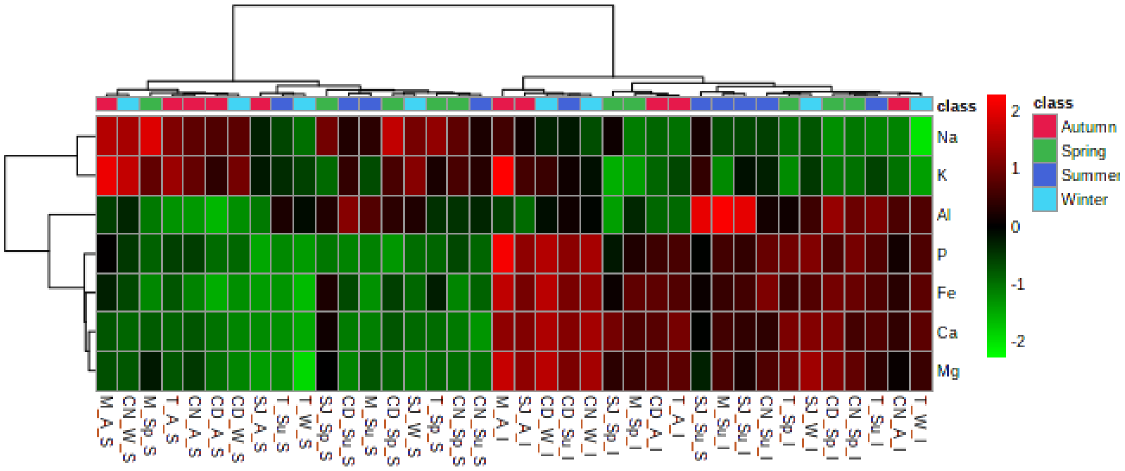


Figure 2. 4 Heatmap comparing major elements bioaccumulation (Al, Ca, Fe, K, Mg, Na and P) in soluble and insoluble fraction of tissue samples. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer. I and S represents the initials for insoluble and soluble fraction.

In the PCO of physical and chemical parameters with the insoluble fraction of tissue organisms, axis 1 explained 30.6% of total data variation separating Winter, site CD, site SJ and site T of Autumn, site CD and site CN of Spring and Summer in the positive side. These sites were associated with a higher correlation (r> 0.7) of the major elements' Ca, Fe, and P and with DO, % fines, pH and % OM with this axis. Site M and site CN of Autumn, site M, SJ and T of Spring and Summer were separated in the negative side. This axis was negatively correlated (r> 0.7) with the abiotic factors' salinity and Eh (Figure 2.5).

Axis 2 explained 16.2% of total variation separating Winter, site SJ of Autumn, site M and T of Spring and site M, SJ and T of Summer in the positive side. The concentrations of the major elements Ca, Fe, Mg and P and higher Eh were positively correlated (r> 0.7) with this axis. In the negative side of axis 2 were separated the sites CD, M, SJ and T of Autumn, sites CD, SJ and CN of Spring and sites CD and CN of Summer. Salinity, % OM, % fines, DO and pH were negatively correlated with this axis (Figure 2.5).

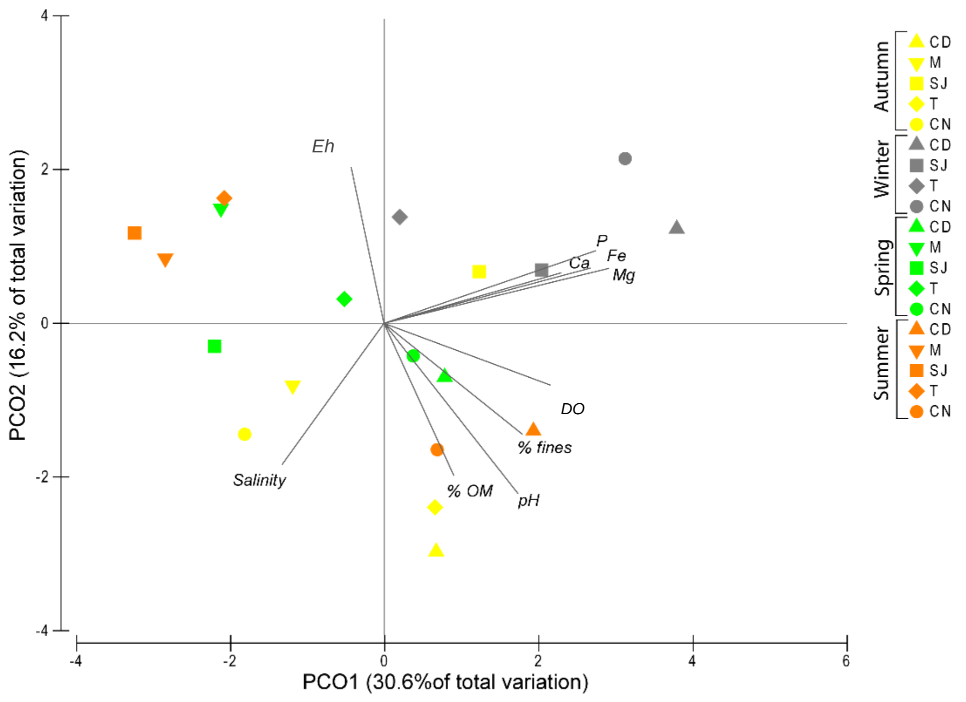


Figure 2. 5 Principal coordinate ordination analysis (PCO) of the insoluble fraction of *Diopatra neapolitana* tissues and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely major elements bioaccumulation data and physical and chemical parameters (r> 0.7): Ca; Fe; Mg; P; DO; pH; % fines; % OM; Salinity; Eh. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

Regarding the PCO of physical and chemical parameters with the soluble fraction of tissue organisms (Figure 2.6), axis 1 explained 28.1% of total data variation separating Winter, Autumn (except site M), site CN of Spring, and site CD and T of Summer in the positive side. DO, % of fines and pH had a positive correlation with axis 1. Site M of Autumn, Spring (except CN) and site M and SJ of Summer were on the negative side. It was observed a negative correlation of the major elements Ca, Fe, K, Mg, Na and P and % fines, DO and higher value of pH with this axis (Figure 2.6).

Axis 2 explained 22.1% of total data variation separating site SJ of Autumn, site T of Winter and sites M, SJ and T of Spring and Summer in the positive side. In the negative side were separated samples from Winter (except T), Autumn (except SJ) and sites CD and CN of Spring and Summer. Major elements Ca, Fe, K, Mg, Na and P and physical and chemical parameters DO, % fines and pH were negatively correlated with this axis. (Figure 2.6).

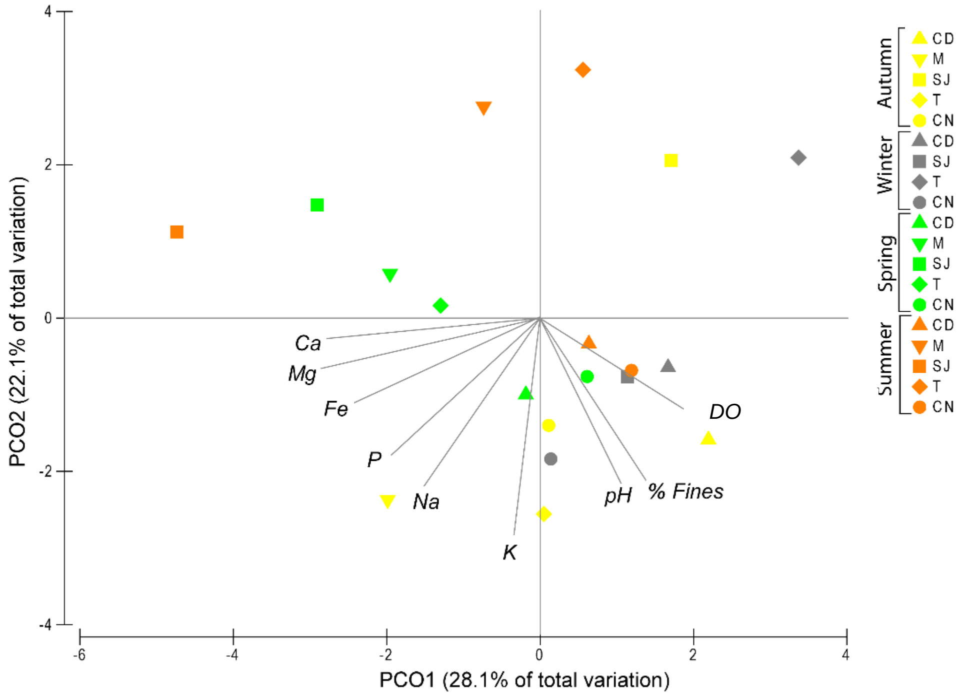


Figure 2. 6 Principal coordinate ordination analysis (PCO) of the soluble fraction of *Diopatra neapolitana* tissues and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely major elements bioaccumulation data and physical and chemical parameters (r> 0.7): Ca; Fe; K; Mg; P; DO; pH; % fines. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

3. Trace elements

3.1 Sediments

Regarding trace elements concentration in the sediments, generally, their concentration was higher in Autumn and lower in Winter and Spring. Site T in Winter had the lowest concentration of trace elements and site CD in Autumn has the highest concentration (Figure 2.7). Trace elements concentration detected in the sediments for each site and season are presented in Figure S2.4. The most contaminated site was CD.

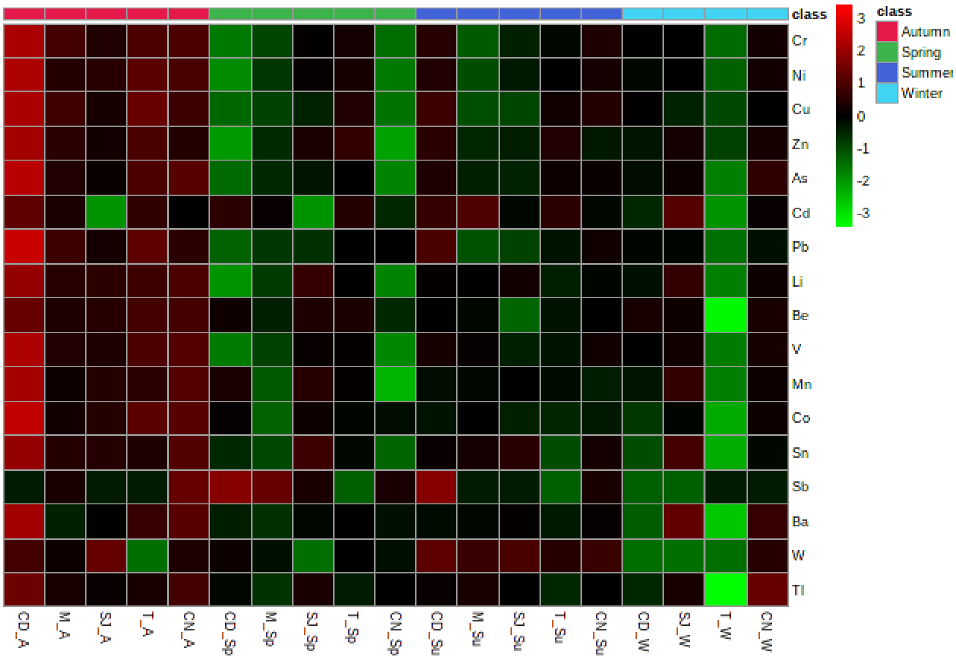


Figure 2. 7 Heatmap comparing trace elements concentration (As, Ba, Be, Cd, Co, Cr, Cu, Li, Mn, Ni, Pb, Sb, Sn, Tl, V, W and Zn) in sediments of sampling sites. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

Regarding the PCO of physical and chemical characteristics and trace elements of sediments, axis 1 showed a 56.7% of total data variation separating, sites from Summer and Spring (except site CD), site SJ of Autumn and sites CD and T of Winter in the positive side, and the remaining in the negative side. Trace elements As, Ba, Be, Co, Cr, Cu, Li, Mn, Ni, Sn, Tl, V and Zn and % of fines, % of OM were negatively correlated with axis 1. (Figure 2.8).

PCO axis 2 showed 10.9% of total data variation separating Winter, site T of Autumn, site CD and CN of Spring and site CN of Summer in the positive side from the remaining that are in the negative side. DO, % fines, % OM and trace elements like Cu, Cr, Mn, Ni, Pb, V and Zn were strongly correlated with this side of the axis 2. The trace elements As, Ba, Be, Co, Li, Sn, Tl, and W and temperature were negatively correlated with axis 2 (Figure 2.8).

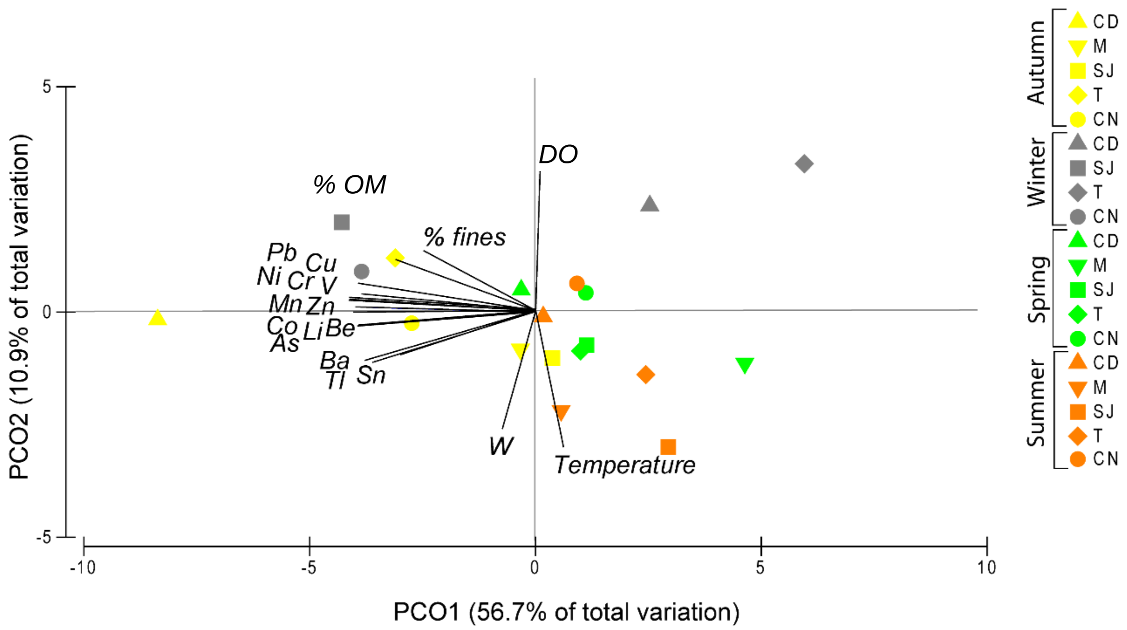


Figure 2. 8 Principal coordinate ordination analysis (PCO) of the sediments collected in sites and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely trace elements concentration data and physical and chemical parameters (r> 0.7): As; Ba; Be; Co; Cr; Cu; Li; Mn; Ni; Pb; Sn; Tl; Zn; W; DO; % fines; %OM; Temperature. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

3.2 Tissue samples

Regarding bioaccumulation of trace elements in polychaetes samples, it was possible to observe a higher bioaccumulation in the insoluble fraction of tissues regardless of seasons and sites, comparing with soluble fraction. However, site SJ in Spring and Summer had high concentrations of trace metals both in soluble and insoluble fraction (Figure 2.9). Be element value was under the detection limit, therefore, is not represented in the heatmap. Overall, Zn was the element that organisms accumulated the most. The site where organisms accumulated the most was in SJ and the season where that accumulation occurred was mainly during Autumn and Summer (Figure S2.5 and Figure S2.6).

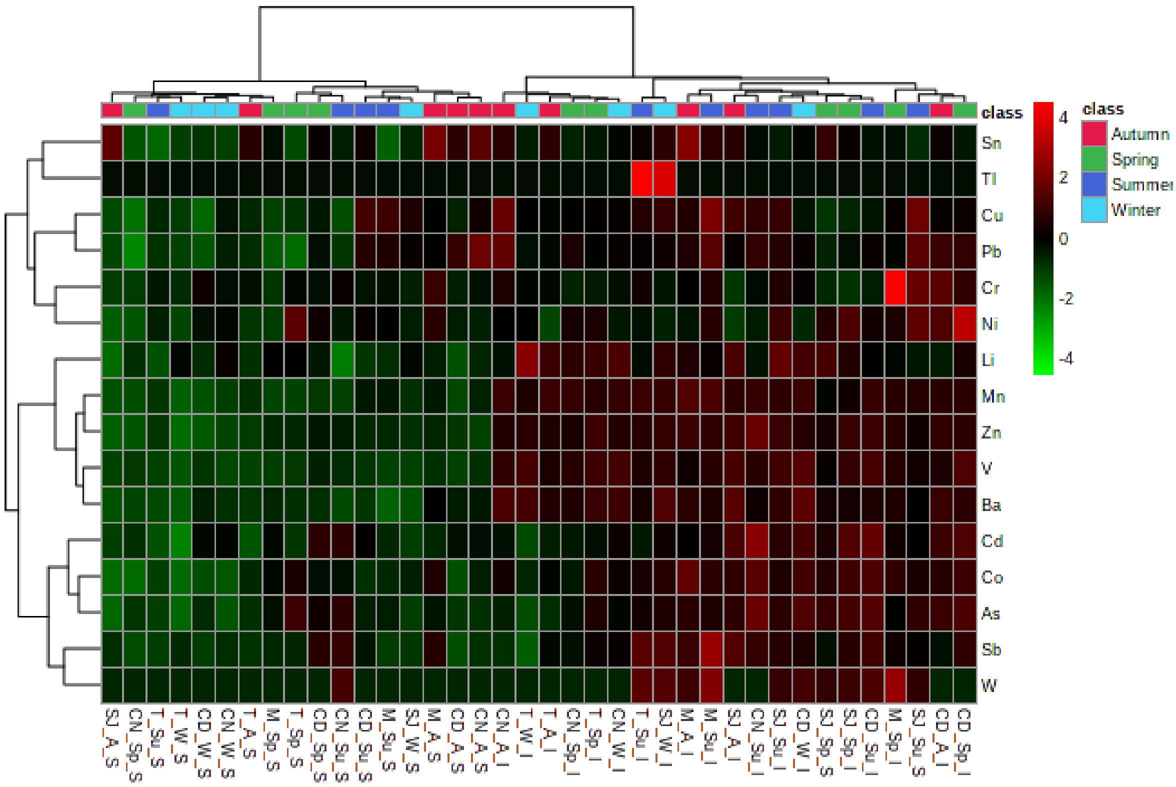


Figure 2. 9 Heatmap comparing trace elements bioaccumulation (As, Ba, Cd, Co, Cr, Cu, Li, Mn, Ni, Pb, Sb, Sn, Tl, V, W and Zn in soluble and insoluble fraction of tissue samples. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer. I and S represents the initials for insoluble and soluble fraction.

Regarding the PCO that compares the physical and chemical parameters with the insoluble fraction of tissue organisms, in axis 1 explained a 22.9% of total data variation separating samples of Summer, site M of Autumn and sites CD, M and site SJ of Spring in the positive side of the axis from the remaining samples. Trace elements As, Co, Cd, Sb and W and temperature and salinity were positively correlated with axis 1 (Figure 2.10).

PCO2 axis, explained 17.5% of total variation separating samples from Spring (except site CD), sites T and CN from Winter and sites M, SJ and T of Summer in the negative side. Trace elements Co, Li, Sb and W were negatively correlated with this axis, while % OM, % fines, pH, salinity and trace elements As and Cd were positively correlated (Figure 2.10).

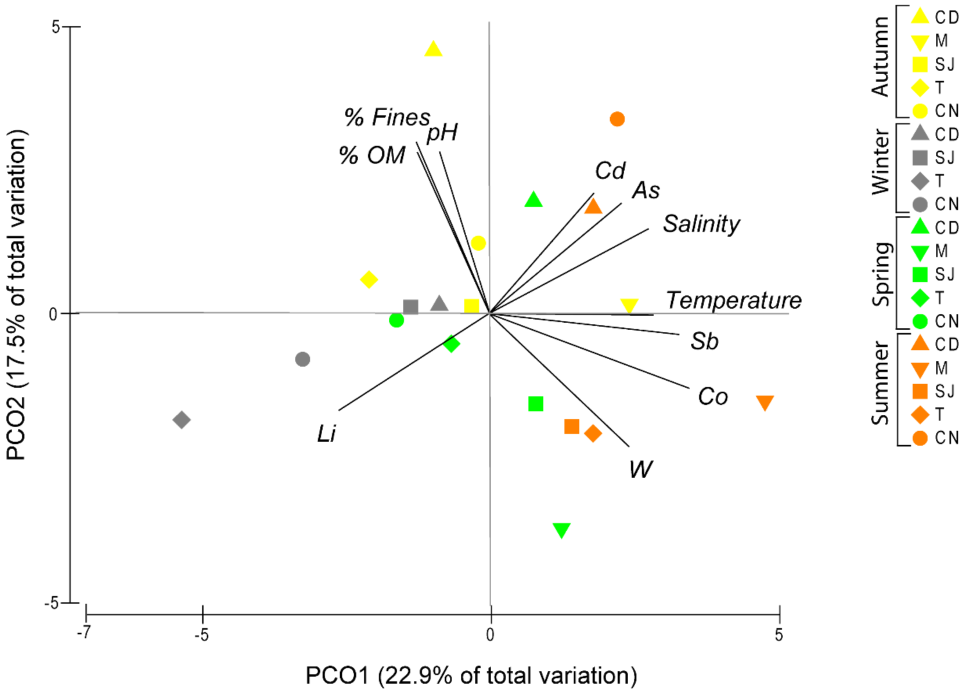


Figure 2. 10 Principal coordinate ordination analysis (PCO) of the insoluble fraction of *Diopatra neapolitana* tissues and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely trace elements bioaccumulation data and physical and chemical parameters (r> 0.7): As; Cd; Co; Li; Sb; W; temperature; pH; % fines; % OM; salinity. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

Concerning the PCO of physical and chemical parameters with trace elements regarding the soluble fraction of tissue organisms, PCO1 showed 31.5% of total variation separating Spring (except site CN), sites M and CN of Autumn and Summer (except site T) in the positive side. The remaining sites were on the negative side, which were correlated (r> 0.7) with higher levels % OM (Figure 2.11).

PCO2 showed a 15.3% of total data variation separating Autumn (except site SJ), site CD and SJ of Winter, site CD of Spring and sites CD and CN of Summer in the positive side. This side was characterized by higher concentrations of Zn being correlated with higher levels of % OM. The negative side was characterized by higher bioaccumulation of As, Co and V (Figure 2.11).

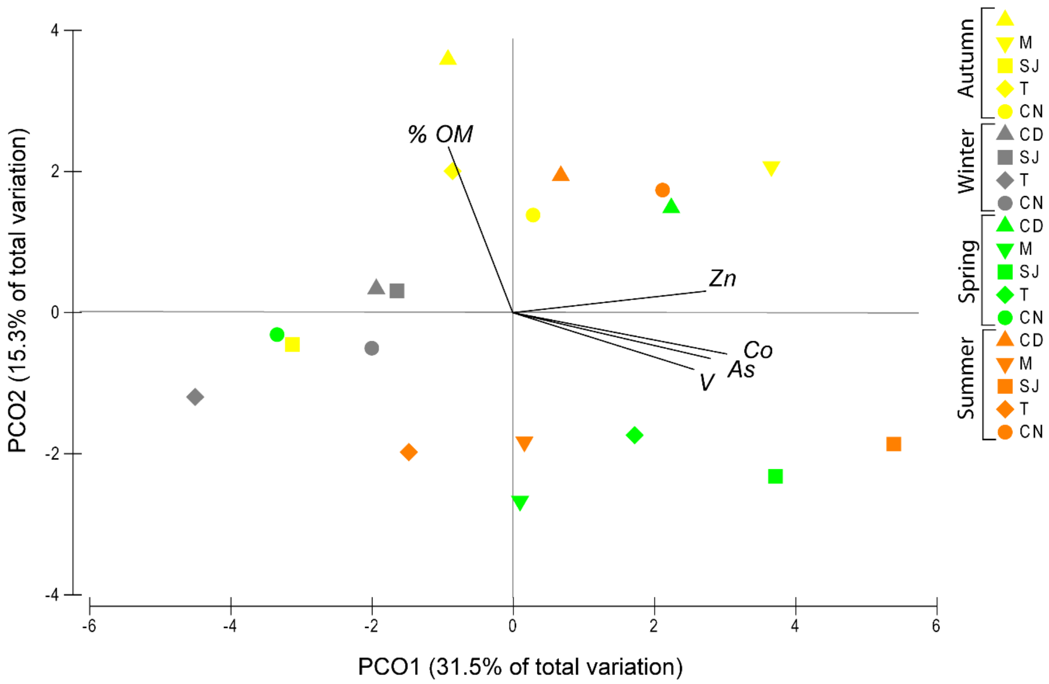


Figure 2. 11 Principal coordinate ordination analysis (PCO) of the soluble fraction of *Diopatra neapolitana* tissues and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely trace elements bioaccumulation data and physical and chemical parameters (r> 0.7): As; Co; V; Zn; % OM. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

Discussion

The ability of polychaetes to bioaccumulate substantial amounts of elements from water and sediments was previously demonstrated in other studies (e.g. [55], [96], [97], [104]). However, there is scarce information about how elements bioaccumulation varies along seasons in polychaetes tissues, neither any of the known studies included a wider variety of elements. Thus, the present work investigated the concentration and bioaccumulation of major and trace elements in the sediments and in *D. neapolitana* specimens' tissues, collected from five sites from Ria de Aveiro lagoon.

Regarding, major and trace elements concentration in sediments, in our study it was demonstrated that, in general, sediments with a higher % OM and % fines presented higher concentrations of major and trace elements. These results lead us to conclude that, in this study, element concentration in sediments is linked to higher levels of % OM and % fines. Elements availability can depend on pH, % OM, salinity, Eh and sediment grain size, and the interaction between these parameters. This might interfere with the solid and solution phases of the elements [105]–[107]. Although elements availability might be related with the parameters mentioned above, this also might be related with seasonal and spatial variation. In our study it was observed a difference of element concentration among areas and seasons, showing that in general, Autumn was the season where higher elements concentration was observed and CD was the site where the elements were observed in higher concentrations. These results allow us to conclude that elements concentration is associated with changes in sediment characteristics amongst locations. Previous works also demonstrated that physical-chemical characteristics of sediment influenced elements concentration in the sediments [55], [108]–[111], some of them conducted at Ria de Aveiro lagoon [55], [109]. However, elements availability also might change due to anthropogenic activity that can alter major and trace elements concentration among areas [10]. Overall, all elements were found in higher concentrations during Autumn in CD, as we mentioned before. This might have happened due to the high % of OM and % fines that occurred in that season.

The major elements with higher concentrations were Al, Ca and Fe (Figure S2.1). The least abundant element was P. Figueira et al., 2011 [112] also observed high levels of Al in sediments from Ria de Aveiro. High levels of Fe might complex to the organic matter leading to high bioavailability of toxic elements [113]. Trace elements that were observed in higher concentrations in all sites were Ba, Cr, Li, Mn, Pb and Zn (Figure S2.4). Similar results were seen by Pires et al., 2017 [55] where the elements in higher concentrations in several areas from Ria de Aveiro were Cr and Pb. Cr and Pb are also the most abundant elements in Óbidos lagoon, and in the Tagus estuary Pb was the most abundant element in sediments [21], [108]. In Venice lagoon, the most abundant elements were also Cr, Pb, and Zn [114]. In this study, the least abundant elements where Cd, Tl and W (Figure S2.4). Other authors also had the same results concerning the low concentration of Cd, in Morrocan estuaries [115] and in the Guadalquivir river estuary [104].

Regarding elements bioaccumulation in tissue samples, the elements that bioaccumulated more in the insoluble and soluble fractions were not the same elements in higher concentrations in the sediments. Regarding major elements, site M during Autumn had higher levels of major elements comparing to the remaining sites, however, concerning trace elements, site CN during Summer had higher levels of elements bioaccumulation. These results allowed us to conclude that element bioaccumulation in tissue organisms was not linked to the total concentration in sediments but with their availability, as stated previously by others authors [55], [108]. Other authors also observed that elements bioaccumulation in the polychaetes *D. neapolitana* and *Hediste diversicolor* tissues were not also correlated with elements concentration in sediments [55], [89] [104], [116]. The results obtained in this study showed a clear separation between elements bioaccumulation in the insoluble and soluble fraction, and that elements were not absorbed in the same amounts. Wallace and Luoma, 2003 [102] showed that element distribution in organisms occurs in the insoluble and soluble fractions. These authors also stated that insoluble fraction retains more elements in the rich granules and cellular debris while the elements in soluble fraction are in the cytosol as free ions or bound to cytoplasmatic molecules such as metallothioneins and metallothionein like proteins and heat sensitive proteins [117]. However, besides the presence of these metallothioneins, most of the elements present in the soluble fraction are readily available for trophic transfer. Therefore is essential to analyze element bioaccumulation in both fractions separately to understand the interaction between species [96].

Regarding major elements bioaccumulation, soluble fraction accumulated more Na and K while insoluble fraction accumulated more Al, Ca, Fe, Mg and P. Highest levels of bioaccumulation of the last major elements mentioned above occurred during Winter and in this season was registered the lowest levels of salinity leading us to conclude that the bioaccumulation of these elements was correlated to low levels of salinity, % OM and % fines of sampling sites. These results indicates that higher bioaccumulation levels in tissues insoluble fraction occurred when low salinities were observed and sediments presented lower % fines and % OM, suggesting that major elements availability was higher in this season. Therefore, the difference of elements bioaccumulation in both fractions is in accordance to the findings that also stated that elements availability depends on physical and chemical factors present in the environment [118] and because of the metallothioneins present in the organisms justifying the fact that Al, Ca, Fe, Mg and P might accumulate more in insoluble fraction and Na and K accumulate more in the soluble fraction. Also, the bioaccumulation of major elements may be related to the type of element and its function in organisms, which may interfere with the elements' availability [119].

Overall, insoluble fraction bioaccumulated more trace elements than the soluble fraction. Similar to major elements accumulation, trace elements had bioaccumulated more major elements during Winter, when the temperature and salinity were lower. In this season, values of pH, % OM and % fines were also low. pH affects absorption processes and the solubility of metal hydroxide mineral and trace elements solubility increases with the decrease of pH values, leading to more dissolved elements that might become available to be incorporated by organisms [109]. Low levels of pH reported in this study for this season, along with the low temperatures and salinity might justify the accumulation of elements in both fractions. However, soluble fraction did not have significant trace elements accumulation levels, compared to the insoluble fraction. As mentioned above, organisms have metallothioneins that might bond to elements when they are absorbed [102]. These results lead us to conclude that trace elements are more sequestrated, causing less toxicity in organisms and therefore preventing damages caused by these elements [120]. In general, the most accumulated elements were As, Cr, Cu, Mn, Ni and Zn in both fractions (Figure S2.5 and Figure S2.6). Other authors also have reported the presence of these elements in tissue samples from organisms invertebrates (e.g. [91], [121], [122]). However, even in the lowest concentrations, there are some trace elements that can be very toxic to organisms. Although Cd is not one of the most accumulated elements, in low levels it can become very toxic to organisms provoking the production of reactive oxygen species (ROS) oxidative damage to essential macromolecules [123], [124].

In general, Autumn was the season where most of the elements were in higher concentrations in the sediments. However, regarding tissue samples, Winter was the season where higher bioaccumulation of elements like Ca, Cr, Li, K, Tl and V in organisms was observed. In this study, elements accumulation was not directly related to the concentration of elements in the sediments, indicating that factors like physical and chemical features (e.g temperature, salinity, pH, % fines, % OM, DO and Eh) interfere with elements availability. Moreover, it is expected that elements bioaccumulation might interfere in organisms performance as previously observed by other authors (e.g. [55], [89], [97]). Therefore, this study demonstrated that elements bioaccumulation in organisms that lived exposed to them were influenced by seasonality and spatial variation.

Chapter III- *Diopatra neapolitana* performance between seasons- biochemical analysis

Abstract

Polychaetes are known to be good bioindicators of marine pollution, as elements contamination. Elements are pollutants very present in sediments, and since polychaetes live buried in them, these organisms may bioaccumulate these pollutants. *Diopatra neapolitana* species were collected along Autumn, Winter, Spring and Summer of 2018/2019 in Ria de Aveiro to infer what changes these organisms go through and in what season there are most negative impacts in them. Biochemical parameters that were analyzed were protein, glycogen, electron transport system, catalase, superoxide dismutase, glutathione s-transferases, non-protein thiols, lipid peroxidation and protein carbonylation (PROT, GLY, ETS, CAT, SOD, GSTs, NPT, LPO and ProC) and metal quantification using the inductively coupled plasma-mass spectrometry (ICP-MS) method was done in organism tissues. PROT did not presented significant differences, GLY had the lowest levels during Spring and ETS, SOD and LPO the highest during Winter. CAT, GSTs and NPT had high levels in Summer. ProC had the highest levels during Autumn. These interferences occurred in the performance of organisms seemed to be related mainly to temperature, salinity and the accumulation of elements. Autumn and Summer were the seasons where they seemed to be in more stressful conditions.

Keywords: *Diopatra neapolitana*, biochemical analysis, major and trace elements, seasonality

Introduction

Invertebrates, such as polychaetes, are widely used in studies of impact assessment in scenarios of stress conditions due to pollutants or climate change since they play an important role in food chain and some of them are very tolerant to changes in their habitats [29], [125]. Therefore, this group of organisms help us to infer how damaged the environment they live might be.

Polychaetes are usually the most abundant taxon in benthic communities [47], [52] and has a wide geographical range [50], [62], [63]. Their life cycle is not very complex, it is easy to collect and maintain in laboratory conditions and they usually live buried in sediment, making them highly exposed to pollutants [53], [90]. *Diopatra neapolitana* is a polychaete species that in addition to having all the characteristics above mentioned, is also very used in fisheries as a fish bait [50], being economically important. Additionally, this species is also very important to feed many other species, like birds, crustaceans and fishes. This species is mainly sedimentary and inhabits in a tube built by the organism, being most of the time buried in sediment [126]. The tube consists of a secreted layer, to which sand particles, fragments of seashells and algae attach to it [71]. Besides being always in contact with sediment, their feeding strategies also puts them in to danger to absorb other pollutants orally [52].

Pollutants can be from anthropogenic or natural source, however, nowadays, natural pollutants tend to be more toxic to marine organisms since they change their configuration due to physical and chemical changes in waters consequently from anthropogenic activity [11]. Metals are elements that exist naturally in the environment but in certain amounts they can become very toxic and cause deleterious consequences to marine organisms (e.g [127], [128]). Although metals have a natural origin, they also have an anthropogenic origin and this origin can be as varied as mining and smelting operations, refining and electroplating, dye and paint manufacture, and fossil-fuel burning [129]. Organisms that are both in contact with sediment and water, as polychaetes, are more conducive to accumulate these elements and to suffer with physical and chemical changes that occur in their environment [55]. The increase of reactive oxygen species, the inhibition of antioxidant enzymes, decrease in fecundity and mortality are some of the various impacts that metals have in these organisms [130]–[132]

Previous studies indicated that environmental fluctuations, like temperature and salinity had impacts on polychaetes [67], [68], [94]. Thus, it is expected that on environmental programs in ecosystems with low levels of pollution could be difficult to determine if the observed effects are due to contaminants or to natural changes closely linked to the life cycle of the organism [55], [133]. Therefore, it is important to understand how natural variations associated with seasonality, such as temperature and salinity will impact the life cycle of benthic fauna and consequently alter the organism's responses to contaminants.

Organisms responses to stressful conditions can be measured at the sub-cellular level, trough biochemical analysis. These responses can offer sensitive information about toxic impacts on organism health and allow to observe early signs of biological response to contaminants [134]–[136].

Therefore, in this study, we pretended to evaluate the impact of elements contamination in *D. neapolitana* species harvested in different sites of Ria de Aveiro lagoon, along the four seasons of the year, also testing the hypothesis that seasons have effects on polychaetes performance. For this, the biochemical performance of *D. neapolitana* organisms (membrane related parameters, antioxidant defenses and oxidative damages), collected in five different areas of Ria de Aveiro lagoon with different levels of elements contamination, was assessed during four seasons (Autumn, Winter, Spring and Summer).

Methodology

1. Study area and field sampling

During the Autumn of 2018 and the Winter, Spring and Summer of 2019, polychaetes were collected from five different sites (Cale Do Ouro- CD, Murtosa- M, São Jacinto- SJ, Torreira- T and Costa Nova- CN) of Ria de Aveiro. The Ria of Aveiro is a shallow coastal lagoon located on the Atlantic coast of northwest Portugal (40'38'N, 8'44'W), separated from the sea by a sand bar, and characterized by narrow channels and the existence of significant intertidal areas, namely mudflats and salt marshes [16]. Collected polychaetes were transported in ice-cold plastic containers to the laboratory and were frozen at -20 °C until further analysis (biochemical endpoints and elements bioaccumulation).

2. Biochemical analysis

Frozen specimens (9 organisms per area, for each season) were homogenized with a mortar and a pestle with liquid nitrogen and divided into subsamples. Extraction was performed with a specific buffer for each biochemical parameter in the proportion 1:2 w/v. Samples were extracted with phosphate buffer 50 mM potassium phosphate (pH=7), 1 mM ethylenediamine tetra acetic acid (EDTA), 1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), for protein (PROT), glycogen (GLY), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferases (GSTs), non-protein thiols (NPT) and protein carbonylation (ProC). For electron transport system (ETS) determination, samples extraction was done in 0.1 M Tris–HCl pH 8.5, 15% (w/v) polyvinylpyrrolidone (PVP), 153 μM magnesium sulphate ($MgSO_4$) and 0.2% (v/v) Triton X-100. Extraction with 20% (v/v) trichloroacetic acid (TCA) was performed for lipid peroxidation (LPO) determination.

Samples were centrifuged for 20 min at 3000 g at 4 °C for electron transport system, and the remaining samples were centrifuged for 20 min at 10 000 g, at 4 °C. Samples for GLY and NPTs determination were not centrifuged. Supernatants were stored at −20 °C or immediately used. All biochemical parameters were determined in duplicate.

2.1 Metabolism related parameters

2.1.1. <u>Protein (PROT)</u>

This biochemical parameter was measured following the method of Biuret described by Robinson and Hogden, 1940 [137] using bovine serum albumin (BSA) as standard (0-40 mg/mL). The samples and standards were incubated in the dark during 10 min at 30 °C with Biuret reagent.

The absorbance was read at 540 nm and the total content was estimated through the standard curve expressed in mg/g of fresh weight (FW).

2.1.2. <u>Glycogen (GLY)</u>

The quantification of GLY was followed by the method described by Dubois et al (1956) [138] using phenol and sulfuric acid. Glucose standards concentrations used were 0; 0.1; 0.4; 1; 2; 3; 4; 5 and 6 mg/mL. First, the samples were pipetted to microtubes with phenol and sulfuric acid and incubated during 30 min at room temperature in a fume hood. Next, the 300 µl of the microtube content was transferred to the 96-well microplate.

The absorbance was read at 492 nm and the results were expressed in mg/g of FW.

2.1.3. <u>Electron transport system (ETS)</u>

ETS activity was measured following the method described by King and Packard (1975) [139], with the modifications performed by De Coen and Janssen (1997) [140]. Balanced salt solution (BSS) buffer 0.13 M Tris-HCl 0.3% (v/v) Triton X-100 pH 8.5, NADH 1.7 mM and NADPH 250 µM and p-iodonitrotetrazolium 8 mM were mixed with the samples. Absorbance was read at 490 nm during 10 min with 25 s readings. The amount of formazan formed was calculated using ε = 15.900 M^{-1} cm^{-1} and the results were expressed in nmol/min per g FW.

2.2 Antioxidant defenses

2.2.1 <u>Catalase (CAT)</u>

CAT activity was quantified according to Johansson and Borg (1988) [141]. Formaldehyde standards (0–150 µM) were used for the standard curve. The samples and standards were incubated for 20 min with 50 mM potassium phosphate buffer (pH 7.0), methanol and 35.28 mM hydrogen peroxide. To stop this reaction, 10M potassium hydroxide was added, followed by 34.2mM Purpald and the mixture incubated for 10 min.

After this, 65.2mM potassium periodate was added, and the mixture left to incubate for 5 min. Following this step, absorbance was measured at 540 nm. The enzymatic activity was expressed in U/g FW (U=1 nmol/min).

2.2.2 Superoxide dismutase (SOD)

SOD activity was determined based on the method of Beauchamp and Fridovich (1971) [142]. SOD standards (0-60 U/mL) were used to the standard curve. The samples and standards were incubated for 20 min with 50 mM potassium phosphate buffer (pH=7.0), reaction buffer (50 mM Tris-HCl (pH=8), 0.1 mM DTPA, 0.1 mM Hypoxanthine), reaction buffer with 68.4 µM nitro blue tetrazolium salt (NBT) and xanthine oxidase (XO) 51.6 mU/mL. Following the incubation, SOD activity was measured spectrophotometrically at 560 nm. The activity of this enzyme was expressed in U/g FW.

2.2.3 Glutathione S-transferases (GSTs)

The activity of these isoenzymes was determined following the method of Habig et al. (1974) [143]. GSTs catalyze the conjugation reaction of the CDNB (1-chloro-2,4-dinitrobenzene) substrate with GSH (glutathione), forming a thioether. A reaction solution constituted by potassium phosphate buffer 0.1 mM pH=6.5, glutathione solution (GSH) 10mM and 1-chloro-2,4- dinitrobenzene (CDNB) 60 mM was pipetted into the samples.

This reaction was measured by following the increasing absorbance at 340 nm, taking readings every 15 s until 5 min. GSTs activity was expressed in U/g FW using $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$.

2.2.4 Non-protein thiols (NPT)

Non-protein thiols (NPT) were measured according to the method described by Sedlack and Lindsay (1968) [144], adapted by Parvez et al. (2003) [145] and described by Oliveira et al. (2010) [9]. Samples were pipetted with salicylic acid and incubated during 1 h. Then, samples were centrifuged during 20 min at 13400 g. After the centrifugation, samples were pipetted to the 96-well microplate adding sodium phosphate buffer and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB).

Samples absorbance was read at 412 nm and the results were expressed in nmol/g of FW.

2.3 Oxidative damage endpoints

2.3.1 <u>Lipid peroxidation (LPO)</u>

This biochemical parameter was measured according to the method described by Buege & Aust, (1978) [147]. Samples were incubated in microtubes pierced with needles in an oven during 25 min at 96 °C with trichloroacetic acid (TCA) 20% (w/v) and 2-thiobarbituric acid (TBA) 0.5% (w/v).

Samples absorbance was read at 532 nm and the analysis was made by the calculation of malondialdehyde (MDA) concentration using $\varepsilon = 1.56 \times 10^5\ M^{-1}cm^{-1}$. LPO levels were expressed in nmol of MDA formed per g of fresh weight.

2.3.2 <u>Protein Carbonilation (ProC)</u>

This biochemical parameter was measured following the methods described in Mesquita et al (2014) [148] and Udenigwe et al (2016) [149]. Samples were incubated at room temperature during 10 min with 2,4-dinitrophenylhydrazine (DNPH) 10 mM in a plate stirrer. Then, incubated for more 10 min at room temperature with sodium hydroxide (NaOH) 6 M also in a plate stirrer.

The absorbance was read at 450 nm and the protein-conjugated hydrazine was calculated using $\varepsilon = 22.308\ mM^{-1}\ cm^{-1}$. Results were expressed in µmol/g of FW.

3. Statistical analysis

Biochemical descriptors (PROT content, GLY, ETS, CAT, SOD, GSTs, NPT, LPO and ProC) were submitted to hypothesis testing using permutational multivariate analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 [103]. For each descriptor significant differences among areas and among seasons were assessed. All descriptors were analyzed following a one-way hierarchical design, with areas as the main fixed factor. The null hypotheses tested were: a) for each biochemical parameter (PROT, GLY, ETS, CAT, SOD, GSTs, NPT, LPO and ProC), for each season, no significant differences exist among different areas; b) for each biochemical parameter (PROT, GLY, ETS, CAT, SOD, GSTs, NPT, LPO and ProC) of each area, no significant differences exist among seasons. Significance levels ($p \leq 0.05$) among sampling areas and among seasons were presented with different letters.

Data from the biochemical parameters and environmental parameters (pH, salinity, temperature, dissolved oxygen (DO), redox potential (Eh), fines content (% fines) and organic matter (% OM) – chapter II)), per area and per season, were transformed (square root), normalized and used to calculate a Euclidean matrix between sampling areas. A Principal Coordinates Ordination analysis (PCO) was used to visualize differences among areas. The environmental parameters highly correlated ($r > 0.7$) were represented as superimposed vectors in the graph.

Afterwards, the matrix containing data from the biochemical parameters and concentration elements in sediments, bioaccumulation in *D. neapolitana* soluble and insoluble fractions of elements (chapter II) per sampling area and season was used to perform another PCO analysis. The variables presenting a correlation higher than 70 % with samples ordination were represented as superimposed vectors in the PCO graph.

Results

1. Metabolism related parameters

Protein (PROT) content was lower in organisms from site T and CN in Autumn and was higher of organisms from SJ in Spring and CN in Winter (Figure 3.1A; Supplementary table 1).

Glycogen (GLY) concentration for almost all areas were observed significant differences among seasons, with site CN presenting less differences (Figure 3.1B; Supplementary table 1).

In general, electron transport system (ETS) activity decreased in organisms collected in Autumn and Spring for all sites, except in site M and for Winter in CN, where no significant differences were observed (Figure 3.1C; Supplementary table 1).

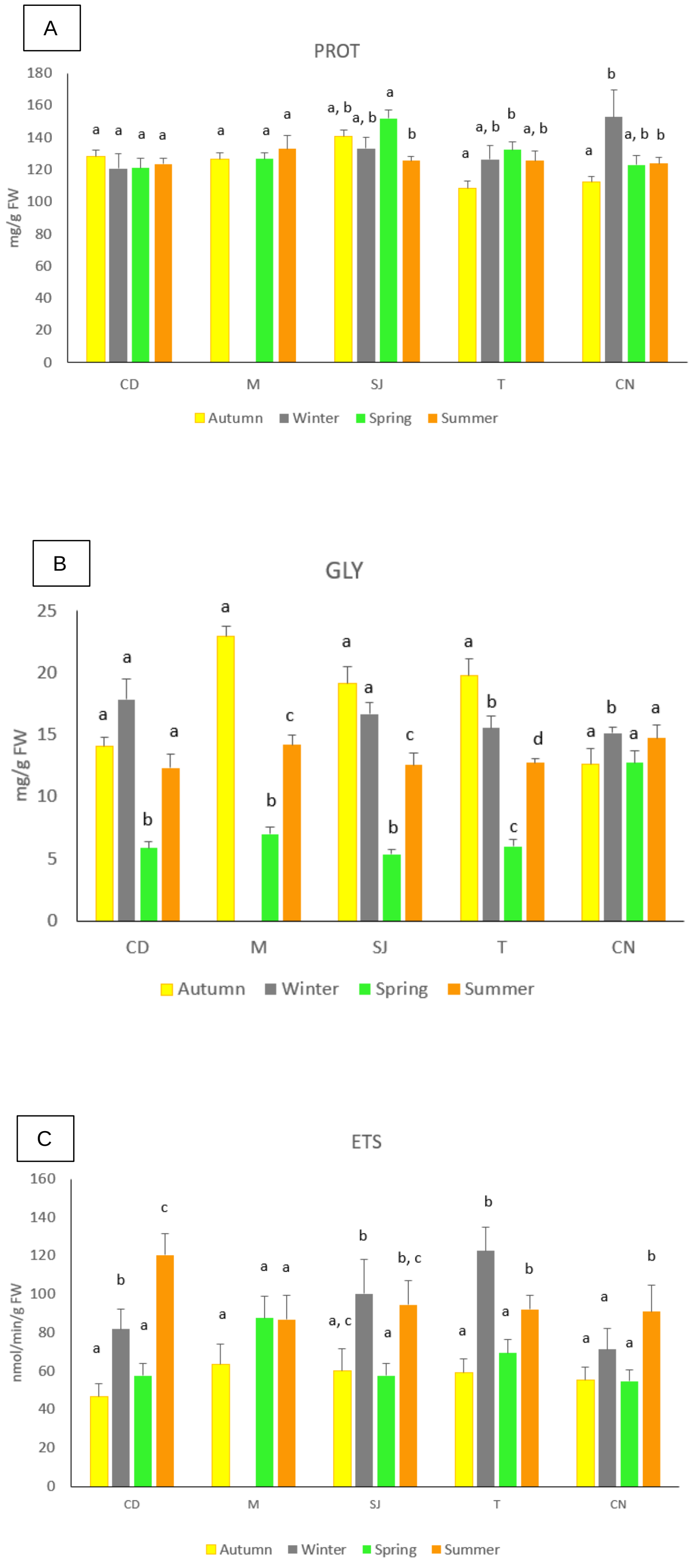


Figure 3. 1 Metabolism related parameters: **A-** protein (PROT), **B-** glycogen (GLY) and **C-** electron transport system (ETS) measured in *Diopatra neapolitana* on Autumn 2018 and Winter, Spring and Summer 2019. Comparison between different seasons on the same local. Letters a, b and c means that exists significant differences between seasons (p≤ 0.05). CD: Cale do Ouro; M: Murtosa; SJ: São Jacinto; T: Torreira; CN: Costa Nova. Site M during Winter does not have data.

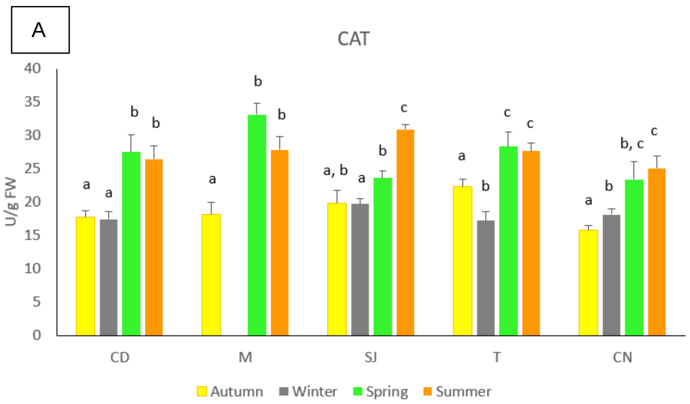
2. Antioxidant defenses

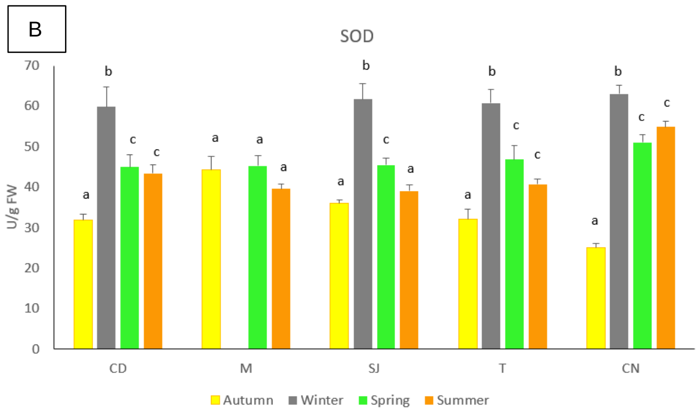
Catalase (CAT) activity generally increased in Spring and Summer in all areas. This increase was significant for all sites and seasons, except in SJ in Autumn and Spring and CN in Winter and Spring (Figure 3.2A; Supplementary table 1).

Superoxide dismutase (SOD) activity generally increased in all sites during Winter and was lower in organisms from CN during Autumn. This increase was significantly different in all sites except in site M. The lower activity during Autumn in CN was also significantly different (Figure 3.2B; Supplementary table 1).

GSTs activity was significantly lower in all sites during Spring and significantly higher during Winter and Summer in site T (Figure 3.2C; Supplementary table 1).

Generally, non-protein thiols (NPT) were higher during Spring and Summer in all sites, except in Costa Nova which during the Spring had a decrease. The lowest value was during Autumn in CD (Figure 3.2D; Supplementary table 1).





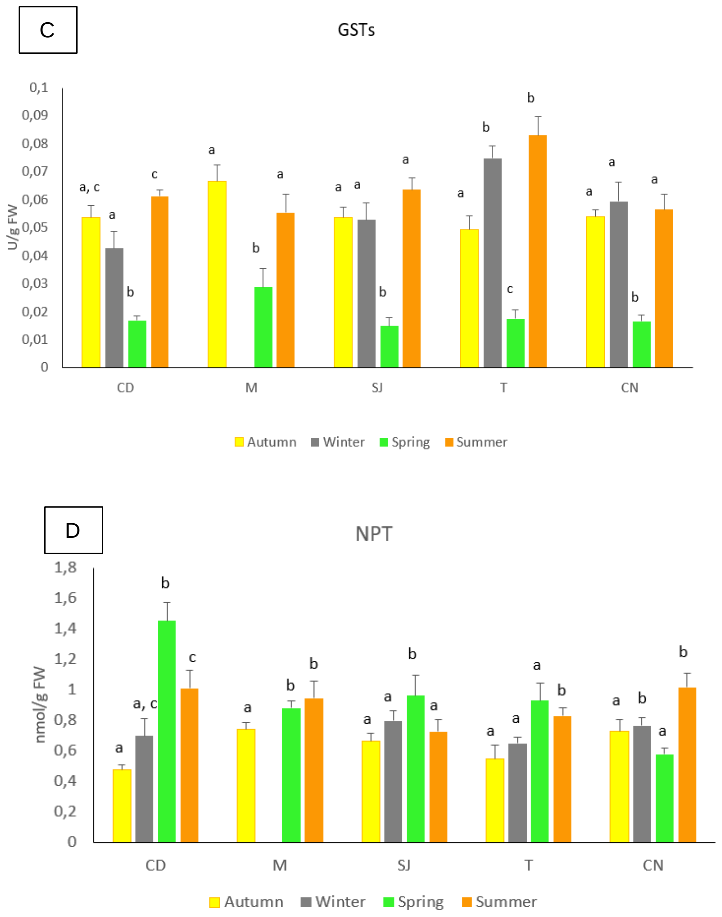


Figure 3. 2 Antioxidant defenses: **A-** catalase (CAT), **B-** superoxide dismutase (SOD), **C-** glutathione S-transferases and **D-** non-protein thiols (NPT) measured in *Diopatra neapolitana* on Autumn 2018 and Winter, Spring and Summer 2019. Comparison between different seasons on the same site. Letters a, b and c represent significant differences between seasons ($p \leq 0.05$). CD: Cale do Ouro; M: Murtosa; SJ: São Jacinto; T: Torreira; CN: Costa Nova. Site M during Winter does not have data.

3. Oxidative damage endpoints

Lipid peroxidation (LPO) levels were significantly higher during Winter, especially in SJ, where all levels of LPO were significantly different among seasons. Also, the lowest levels of LPO were seen in Summer in SJ. Generally, during Autumn LPO levels were low, except in CN which were significantly higher (Figure 3.3A; Supplementary table 1).

ProC was higher in all sites during Autumn except in CD, where no significant differences between seasons were observed. Also, in Autumn, levels were significantly higher in CN. In SJ, during Winter, levels were higher comparing with the other sites (Figure 3.3B; Supplementary Table S1).

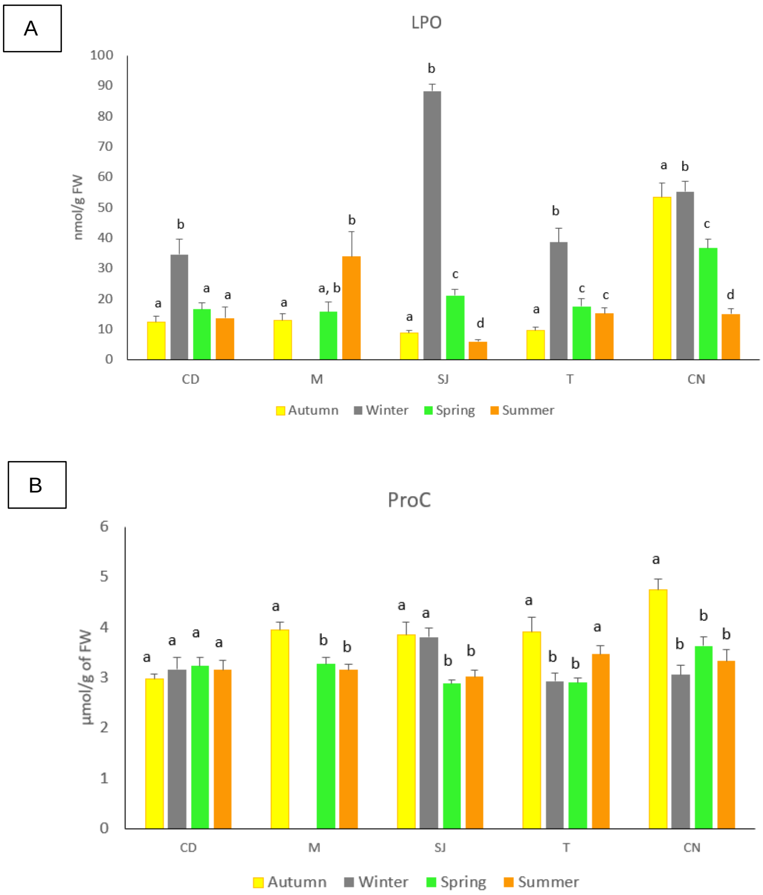


Figure 3. 3 Oxidative damage endpoints: **A-** lipid peroxidation (LPO) and **B-** protein carbonylation measured in *Diopatra neapolitana* on Autumn 2018 and Winter, Spring and Summer 2019. Comparison between different seasons on the same local. Letters a, b, c and d means that exists significant differences between seasons (p≤ 0.05). CD: Cale do Ouro; M: Murtosa; SJ: São Jacinto; T: Torreira; CN: Costa Nova. Site M during Winter does not have data.

4. Biochemical parameters and physical-chemical data

Principal Coordinates Ordination (PCO) evidenced that together PCO1 and PCO2 explained 46.8% of the total variation obtained among environmental data and biochemical parameters among areas (Figure 3.4). PCO1 described 26% of total data variation separating Autumn and Winter (positive side) from Spring and Summer, except site CN (negative side). The results obtained revealed that sampling sites grouped together according to season, with different areas from the same season clustering together. GLY content presented a higher correlation (r> 0.7) with PCO1 positive side. On the other hand, high levels of CAT activity were strongly correlated with negative side of PCO1, as high values of temperature and salinity (Figure 3.4), with polychaetes collected in Spring and Summer presenting higher values of CAT activity. PCO2 showed 20.8% of total variation separating Winter, sites M and SJ of Spring and Summer and site T of Summer in the positive side, presenting a positive correlation with SOD activity. On the other hand, higher CAT activity and higher levels of temperature and salinity were correlated with the negative side of PCO2 (Figure 3.4).

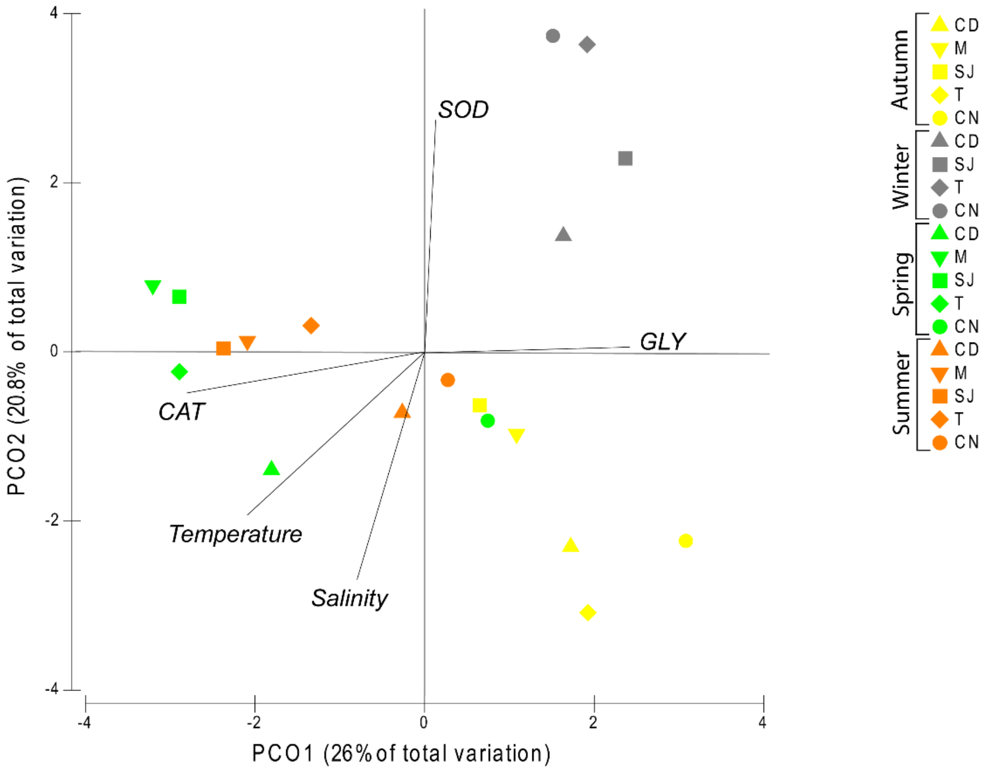


Figure 3. 4 Principal coordinate ordination analysis (PCO) of the biochemical and physical and chemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely biochemical and physical and chemical parameters (r> 0.7): CAT; GLY; SOD; temperature; salinity. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

5. Elements and biochemical parameters correlation

5.1 Major elements

PCO regarding major elements bioaccumulation in insoluble fraction and biochemical parameters evidenced that together PCO1 and PCO2 explained 46.1% of the total variation obtained among areas (Figure 3.5). PCO1 showed a 25.1% of total data variation separating Spring, Autumn (except site SJ), Summer (except site CD) and site T of Winter in the positive side. This side were correlated with higher concentration of CAT activity and ProC. Biochemical markers GLY, SOD and NPT and the major elements calcium (Ca), iron (Fe), magnesium (Mg) and phosphorus (P) were strongly correlated with negative side of PCO1. PCO2 showed a 21% of total variation separating Summer (except site T), Spring, (except site CN), and site CN of Winter in the positive side. Higher activity of SOD, CAT and NPT presented a positive correlation with PCO2. On the other hand, higher concentrations of major elements, GLY and ProC were correlated with the negative side of PCO2 (Figure 3.5A).

PCO regarding major elements accumulated in soluble fraction and biochemical parameters demonstrated that PCO1 showed 27.3 % of total variation separating Spring (except site CN), site CD, site M and site SJ of Summer, site M of Autumn and site CN of Winter in the positive side, with NPT and CAT activity and most of major elements strongly correlated with this side. GLY, GSTs and ProC levels and the major element potassium (K) are correlated with the negative side of PCO1. PCO2 showed 22.8% of total variation separating Autumn (except SJ) and Winter (except T) in the positive side, from Spring (except site CN and M) and Summer (except site SJ) in the negative side. GLY, ProC and the majority of major elements were correlated with the positive side, while CAT, GSTs NPT and aluminum (Al) were correlated with the negative side (Figure 3.5B).

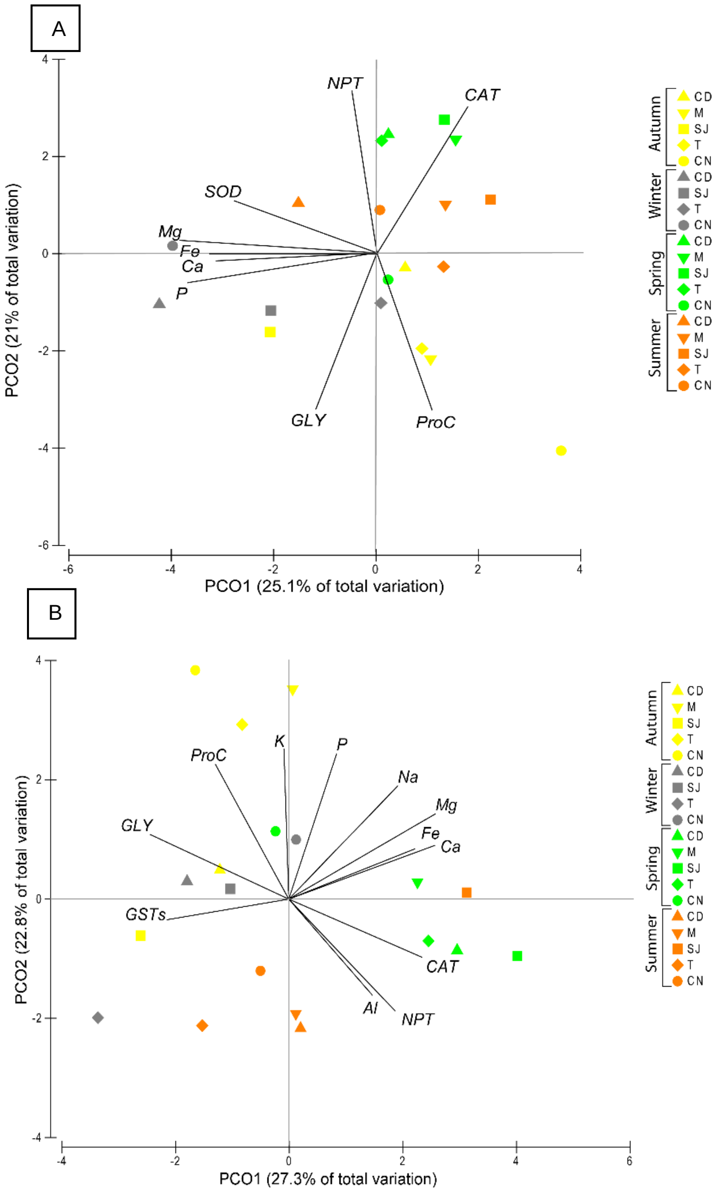


Figure 3. 5 Principal coordinate ordination analysis (PCO) of the insoluble fraction of *Diopatra neapolitana* tissues and biochemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely major elements bioaccumulation data and biochemical parameters (r> 0.7): Ca; Fe; Mg; P; CAT; GLY; NPT; ProC; SOD. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer. **B.** Principal coordinate ordination analysis (PCO) of the soluble fraction of *Diopatra neapolitana* tissues and biochemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely major elements bioaccumulation data and biochemical parameters (r> 0.7): Al; Ca; Fe; K; Mg; Na P; CAT; GLY; GSTs NPT; ProC. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

5.2 Trace elements

PCO regarding trace elements accumulation in the insoluble fraction and biochemical parameters demonstrated that PCO1 explained 21.9% of total variation separating Spring (except CN) and Summer samples in the positive side, and Autumn and Winter samples in the negative side of the axis. PCO2 separated samples from Autumn (except T), Summer and site SJ of Winter in the positive side of the axis, and the remaining in the negative side. From PCO analysis, it was possible to observe that the biochemical markers CAT and NPT were strongly correlated with the positive side of PCO1 and therefore more related with Summer and Spring samples, that presented higher values of arsenium (As), cobalt (Co) and tin (Sn) in the insoluble fraction. In the negative side of PCO1, it was observed a higher correlation of GLY high levels and ProC with higher concentrations of copper (Cu), manganese (Mn), lead (Pb) and Sn in the insoluble fraction of the elements in Summer samples (Figure 3.6A).

Regarding trace elements accumulation on the soluble fraction and biochemical parameters, PCO analysis demonstrated that PCO1 explained 29.9% of total data variation separating Summer (except M and T) and Spring (except CN) samples in the positive side, related with higher trace elements (As, barium (Ba), Co, Cu, nickel (Ni), Pb, vanadium (V) and zinc (Zn)) concentration and NPT activity. In the negative side Winter and Autumn samples were mainly related with the biochemical parameters GLY and ProC. PCO2 explained total 16% of variation separating in the negative side samples from Winter, Spring and Summer (except site SJ), being correlated with As and Ni accumulation levels in the soluble fraction. Samples collected in Autumn and site SJ from Summer were separated in the positive side of this axis, being correlated with Ba, Co, Cu, Pb, V and Zn and GLY and ProC (Figure 3.6B).

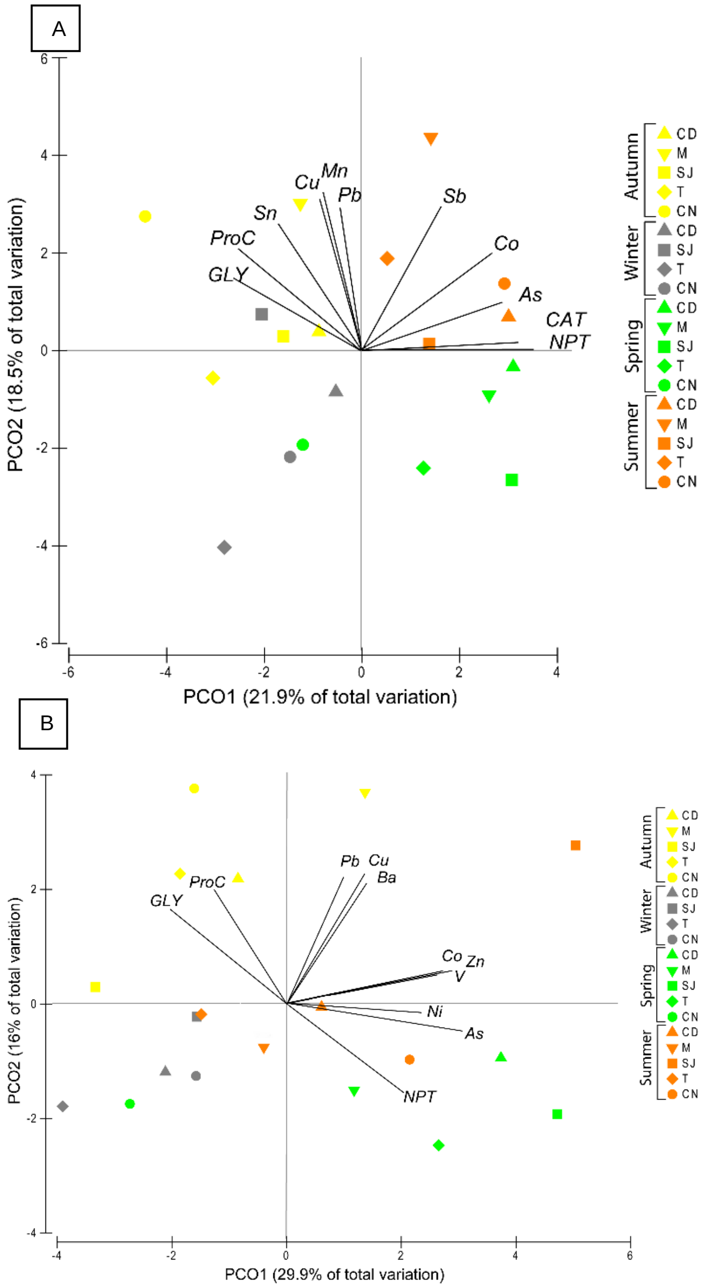


Figure 3. 6 A. Principal coordinate ordination analysis (PCO) of the insoluble fraction of *Diopatra neapolitana* tissues and biochemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely trace elements bioaccumulation data and biochemical parameters (r> 0.7): As; Co; Cu; Mn; Pb; Sb; Sn; CAT; GLY; NPT; ProC. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer. **B.** Principal coordinate ordination analysis (PCO) of the soluble fraction of *Diopatra neapolitana* tissues and biochemical parameters. Pearson correlation vectors are superimposed as supplementary variables namely trace elements bioaccumulation data and biochemical parameters (r> 0.7): As; Ba; Co; Cu; Ni; Pb; V; Zn; GLY; NPT; ProC. CD, M, SJ, T and CN represents sites Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova, respectively. A, W, Sp and Su represents the seasons of the year: Autumn, Winter, Spring and Summer.

Discussion

In this study, *D. neapolitana* organisms were collected from various sites from Ria de Aveiro lagoon over four seasons to try to infer how elements present in the sediments affect organisms from different areas and if these impacts were related with the changes of abiotic factors of seasons. Previous studies demonstrated that high accumulation of metals and metalloids might cause deleterious effects in organisms, such as oxidative stress, which result from the overproduction and accumulation of reactive oxygen species (e.g. [150], [151]). Additionally, other studies revealed that changes on abiotic conditions, such as salinity and temperature, can also impact polychaetes performance [94], [99]. Thus, it is expected that on environmental studies that organisms' performance could be influenced by contaminants but also by natural variations. Therefore, it is important to understand how abiotic factors associated with seasonality, such as temperature and salinity, will impact *D. neapolitana* responses to contaminants.

Proteins play an important role in biological processes and they assist in various chemical processes that occur in living organisms such as repair and maintenance, energy, transport and storage molecules [137]. In our study, protein content had few changes across sites and seasons, and this might have happened because of the variation of sizes of the organisms collected [152]. However, the availability of food might also be related to this slight variation. Helland et al., 2003 [153] showed that the protein content in the copepod *Calanus finmarchicus* females did decrease with the starvation of the organisms and protein content was also higher in organisms during Spring than Autumn. Overall, in our study, protein content is higher during Spring than Autumn, leading us to conclude that these differences might be related to food availability.

GLY is a carbohydrate that plays an important role in energy source and storage and Dubois et al., 1956 [138], described that stressed organisms can decrease their metabolic capacity by avoiding the consumption of energy reserves. The highest values of GLY content were observed during Autumn and Winter and the lowest values during Spring. Higher GLY levels may indicate that organisms did not need to use this energy to activate other metabolic pathways [154]. In this study, at higher temperatures, organisms also tended to accumulate more elements in the insoluble fraction being also observed an increase in GLY content. Thereby, as observed by Fossi et al., 2012 [155], GLY content might not be related to elements bioaccumulation. However, GLY content presented the lowest values in Spring when the temperature starts to increase. These observations may indicate that in this season they must use more energy to activate metabolic pathways, for example, detoxification pathways like antioxidant defenses. Moreover, these low values may be related to their reproduction period, since organisms start to develop gametes in this season [71], [156].

Concerning ETS, which measures the potential metabolic activity in organisms in response to environmental changes (e.g [157]–[159]), the highest values were observed in Winter and Summer and the lowest values were generally in Autumn and Spring. The high values of ETS in Winter may indicate that there was an increase in metabolic activity because organisms were mostly under unfavorable conditions (values of salinity and temperature decreased; high concentrations of major and trace elements like Al, Fe, P, As, Cu, Cr, Ni, Pb and Zn). Overall, Summer also had highest values of ETS activity and high concentrations of trace elements in both fraction of the tissues. This metabolic activity might be related to detoxification pathways, like the increase in SOD and GSTs activity. Moreover, during Summer, there were reported the highest values of temperature and Schmildin et al., 2015 [158] and Morosetti et al., 2020 [83] also had similar results with the amphipod *Gammarus fossarum* and the mussel *Mytilus galloprovinciallis*, where ETS activity increased with the increase of temperature. Autumn was the season with the lowest metabolic activity. Moreover, PROT and GLY content were higher during this season, which seems that *D. neapolitana* organisms reduced their metabolism to preserve energetic reserves [154]. In fact, Sokolova (2013) [154] reported that to fight against oxidative stress situations, organisms (including polychaetes) may reduce their metabolism to preserve their energy reserves.

CAT is an antioxidant enzyme that is present in the peroxisome of cells and is responsible for the degradation of hydrogen peroxide (H_2O_2), converting it into water (H_2O) and oxygen (O_2) [160], and, in general, in our study, this enzyme activity was higher in Spring and Summer. Generally, in these seasons, the temperature and salinity were higher than the others, so a high correlation was observed with higher CAT activity and salinity and water temperature increase (Figure 3.4). Moreover, during these seasons, there was high bioaccumulation of As and Zn in all sites in both fractions, which might be also associated with the increase of the activity (Figure S2. 5, Figure S2.6, Figure 3.4). Pires et al., 2017 [55] also observed an increase on CAT activity in the same species with higher bioaccumulation of As, indicating that the activity of this enzyme increased in the presence of this contaminant. Moreover, Andrade et al. 2019 [161] had similar results with the mussel *Mytilus galloprovincialis* when exposed to warming waters. Although in Autumn and Winter

CAT activity was low, comparing to other seasons, SOD activity was higher mainly during the Winter season.

SOD is an enzyme that catalyzes the dismutation of reactive oxygen species (ROS), the superoxide radical, in H_2O_2 and O_2, providing an important defense against oxidative damage [142]. In Winter season there where high accumulations of major and trace elements in the insoluble fraction of the tissues and in figure 3.4 SOD vector is on the opposite side of the temperature vector, leading us to conclude that this enzyme activity was related not only with the accumulation of elements but also with the decrease of temperature. Other authors also had these findings relating high SOD activity and elements accumulation (e.g [162], [163]) with polychaetas *Hediste diversicolor* and *Sabella spalanzanii.* Bhuiyan et al., 2021 [125] also observed that low temperatures also increased the activity of this enzyme in *Hediste diversicolor.* In this study, Winter was also the season where was recorded the lowest values of salinity. These low values associated with the high SOD activity in this season led us to conclude that this enzyme activity was also related with the decrease of salinity. Freitas et al., 2015 [94] also had similar results with the polychaeta *D. neapolitana*, where in the lowest salinity the SOD activity was the highest.

The enzymes GSTs are a group of isoenzymes involved in the second phase of the xenobiotic biotransformation process [143]. In our study, the highest values of this enzyme activity were recorded mainly during Summer. Figure 3.5B showed the GSTs vector on the opposite side of the major elements' vectors, leading us to conclude that in this study, besides this enzyme activity, elements bioaccumulation may have been lower. However, GSTs activity are also dependent of concentrations of dissolved oxygen [163], [164]; therefore, the highest levels of these enzymes activity might be related with the higher levels of dissolved oxygen, preventing the cellular damage, justifying the low levels of LPO. Additionally, high levels of salinity might seem related to the highest levels of GSTs activity. Magalhães et al., 2019 [29], observed high GSTs activity and low LPO levels in the polychaeta *Nephtys cirrosa* when they were under high salinities concentration.

Similar to CAT, NPT levels were higher mostly during Spring and Summer. Levels of NPT seem to be related with elements concentration and higher temperatures. They participate in many cellular reactions searching for ROS directly and indirectly through enzymatic reactions [165], therefore, these higher levels of NPTs and CAT show us that there are higher production of ROS due to elements accumulation and higher temperature.

In relation to membrane damage, LPO is the oxidation of lipids through ROS, which have targets directly on the membrane's polyunsaturated fatty acids [147]. The highest levels of lipid peroxidation occurred in Winter in all sites, which indicates that it was in this season that there was more damage to membrane levels. Moreover, SOD activity was higher in this period, however, it was not enough to prevent lipid damage. This also may lead us to conclude that lipid peroxidation is linked to lower temperatures and salinity values and lead to membrane damage. A study conducted by Pires et al., 2017 [55] with the same species also observed and increase of LPO levels and SOD activity in organisms with higher trace elements (As and Cu) accumulation. The lowest value of LPO observed was during the Summer at the SJ site and this value may be related to the high activity of CAT and NPT levels, that were able to prevent damage from ROS.

Carbonylated proteins (ProC) are formed when oxidation of amino acid residues occurs and through these carbonyl groups it is possible to determine if there was protein oxidation [148], [149]. In our study, ProC levels obtained did not vary significantly except for Autumn, where the highest values were observed. In this season GSTs levels were also high, which can lead us to conclude that the increase of salinity and elements bioaccumulation might have provoked the production of reactive oxygen species. This phenomenon could lead to irreversible damages and possible cellular death [166], [167]. Morosseti et al., 2020 [83] also did not observed significant changes in ProC in mussel *M. galloprovincialis* exposed to cerium oxide nanoparticles and mercury. Moreover, the same authors also observed a decrease on ETS activity, indicating that low accumulation of elements did not lead to ROS production and organisms continued to use reserve energies and did not damage proteins in the organisms.

According to PCO related to soluble fraction of major elements, NPT and CAT activity are enhanced in Spring and Summer and the major element present in those seasons is Al, which corroborates the idea that the activity of these enzymes is related to the presence of this element. Although Ca, Fe and Mg had higher levels in Winter in insoluble fraction, these elements had higher levels during Spring and Summer in soluble fraction of the tissues. This might indicate that these elements bioaccumulate most in the cytosol than in the membrane when temperature is higher, causing deleterious effects, since the bioaccumulation in the cytosol can be toxic and enter in the food chain, causing toxicity to other organisms [55].

Trace elements majorly are more but in a smaller quantity. However, these smaller quantities can be more toxic than major elements [168]. In general, organisms bioaccumulated more trace elements in Spring and Summer, both in the soluble and insoluble fractions, like major elements, being correlated with higher values of CAT and

NPT in these seasons. These elements entering in the cytosol can be very toxic for the organisms, and so, they must activate antioxidant defenses to eliminate ROS. In Autumn, both fractions had higher bioaccumulation of Cu, Mn, Pb and Sn and also shows higher levels of GLY and ProC.

In general, bioaccumulation by elements can trigger a reaction that allows organisms to detoxify these components, so that they can survive in their environment. This study showed that between major and trace elements, there is no group that accumulates the most since both elements accumulate and generally the most significant bioaccumulation in tissues occurs in the same seasons. The soluble and insoluble fraction do not bioaccumulate elements in the same way, however, they contain cell detoxification pathways.

Chapter IV- Final considerations

Major and trace elements are naturally present in the environment; however, they can cause toxicity to organisms in higher levels.

This study had the objective to characterize, in terms of elements concentration, the sediments of five different sites of Ria de Aveiro along the four seasons of the year and to determine elements bioaccumulation in *Diopatra neapolitana* tissues, how it varies among seasons and what impacts that elements bioaccumulation might have in organisms. Higher elements concentration in sediments occurred when there were higher percentage of fines (% fines) and organic matter (% OM). Overall, the results obtained in this work demonstrated that Autumn was the season where elements concentrated the most in sediments, especially in Cale do Ouro. However, regarding tissue samples, Summer was the season where organisms accumulated more elements and this bioaccumulation occurred when the % fines, % OM, salinity and temperature in their highest. Also, Winter was the season where occurred higher bioaccumulation of elements, due to the lowest levels of salinity and temperature, however, the elements most accumulated during Summer and Winter were different. These results also indicate that these abiotic factors influence elements availability, as previously reported by other authors.

Moreover, elements accumulated differently among fractions in the polychaete’s tissues where insoluble fraction bioaccumulated more elements than soluble fraction. Regarding antioxidant defenses, in general, higher enzyme activities were observed during Spring and Summer due to high temperatures and element bioaccumulation. Metabolism related parameters had higher levels in Spring and Autumn, mainly due to elements bioaccumulation. Oxidative damage was higher during Winter, and this occurred mainly because the physical and chemical characteristics of sampling sites, as salinity and temperature decreased. Also, temperature and salinity were associated to influence the performance of the organisms.

This type of studies involving elements accumulation during different seasons are important because they show how these organisms respond during the changes that occur during the year, since these abiotic factors influence elements geochemistry as well organism’s performance.

Overall, abiotic factors significantly affect organisms in low-contaminated systems, such as Ria de Aveiro which was demonstrated in this study. However, we were able to notice that the concentration of elements in sediment and bioaccumulation in tissues also varied throughout the year, and that in some cases like Summer, some elements bioaccumulation, like As, Mn, Zn, Al and P, also influenced the performance of organisms.

Usually, variables related with seasonality are studied under laboratory conditions, where the conditions from the field are recreated leading to studies that provides us good information about the environment and tolerance of organisms under stressful conditions. Nevertheless, these studies cannot predict all the changes that an environment can suffer among the seasons, becoming difficult to infer what influences the environment and the organisms' health. Therefore, it is challenging to create a pattern linking the organisms' responses to physical and chemical parameters or even element bioaccumulation.

More studies like this should be done, however, not only for one year but also for a more extended period to help to understand how the elements concentrations varies in sediments and elements bioaccumulation in organisms and to assess how the physical-chemical parameters of the collection zones vary among years. Additionally, it would also be curious to compare these variables in other areas of Portugal, or even in the Iberian Peninsula. Also, it could be interesting to study elements availability and correlate with elements bioaccumulation and abiotic factors. In fact, in this study, we detected the elements concentration and we saw that the concentrations varied among the seasons, indicating that some elements were probably dissolved in the water column, becoming more available to the organisms, therefore increasing their bioaccumulation. If elements availability could be done, it could give information about when the elements are available and if our species accumulate them.

Chapter V- References

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Annex

Chapter II- Supplemental files

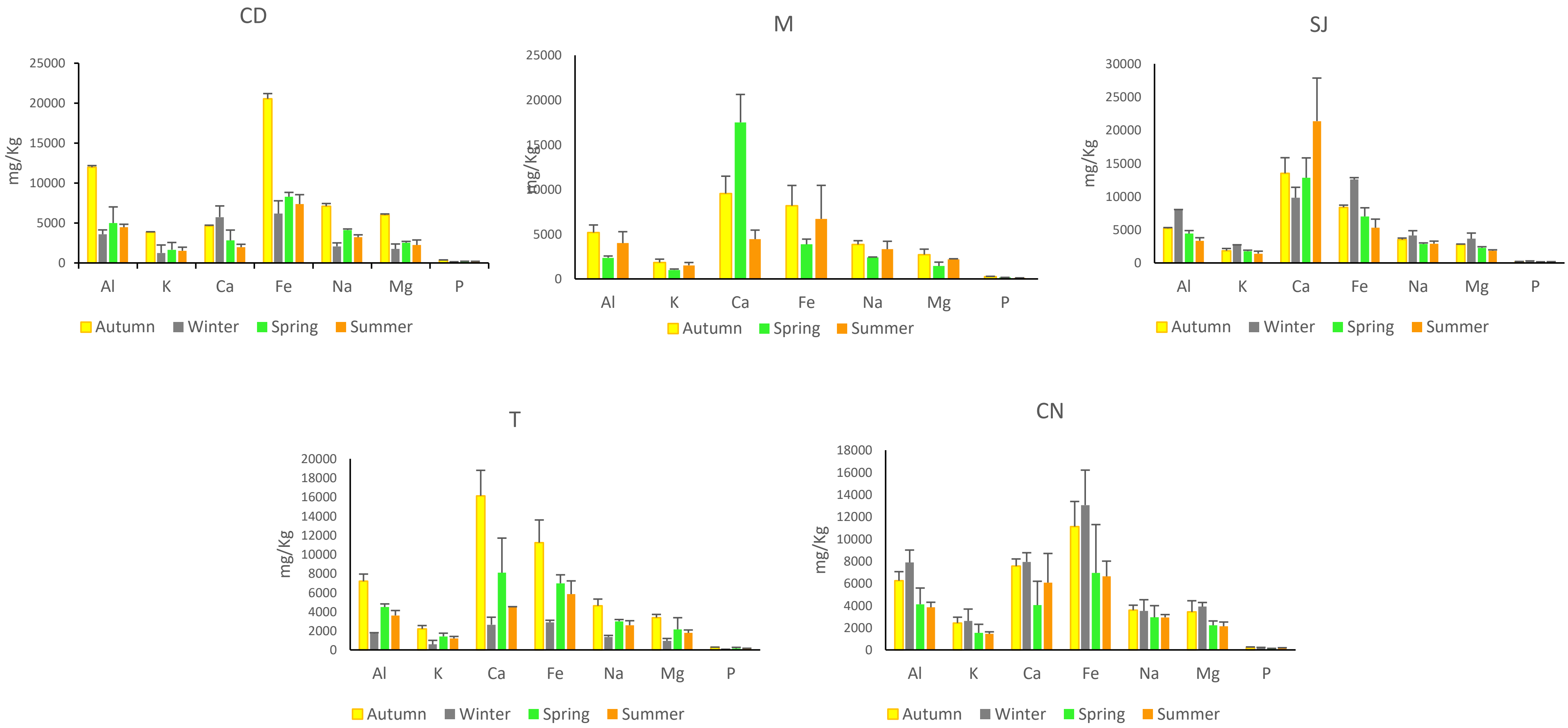

Figure S2. 1 Major elements concentration (aluminium (Al), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na) and phosphorus (P)) measured in sediments collected among Autumn of 2018, Winter, Spring and Summer of 2019 in sampling sites. Initials CD, M, SJ, T and CN stand for Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova.

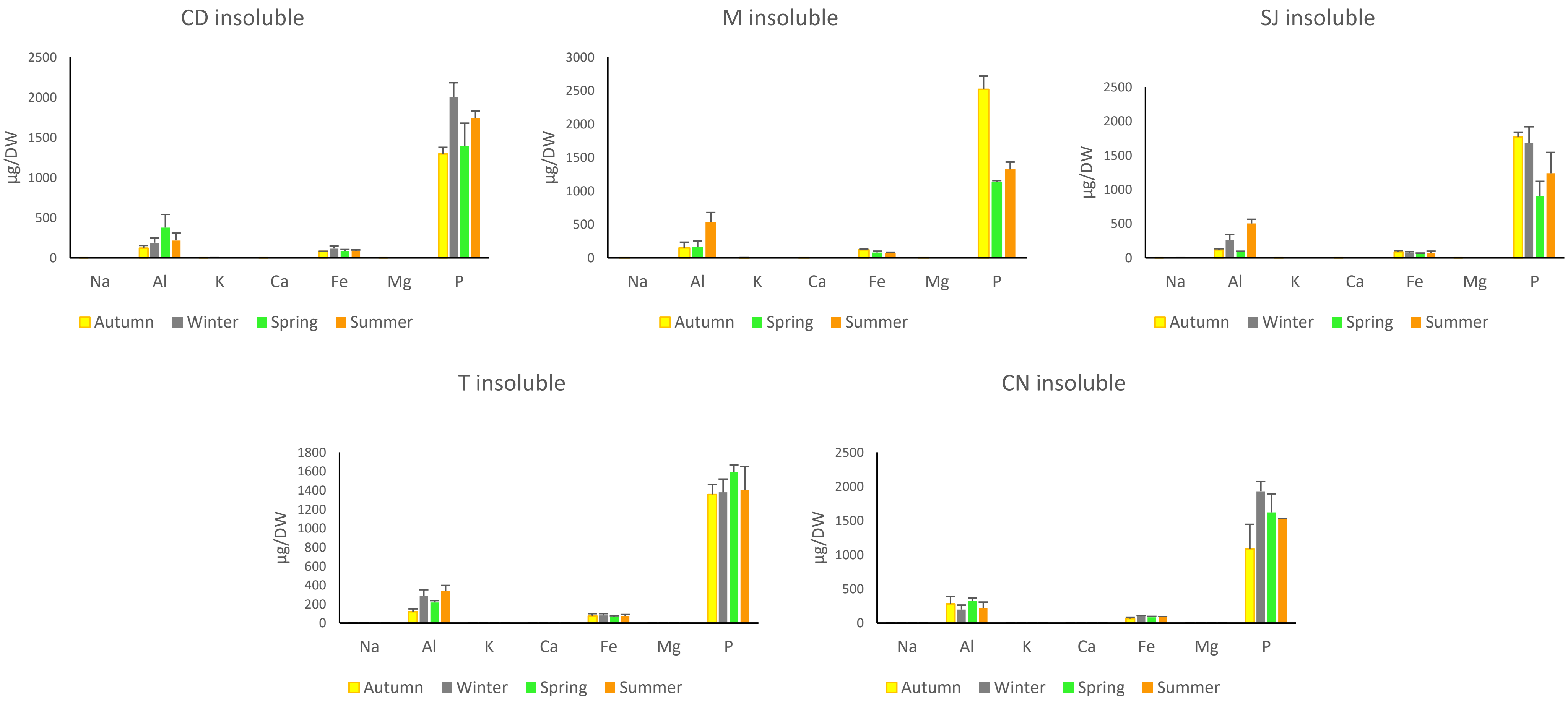

Figure S2. 2 Major elements bioaccumulation (aluminium (Al), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na) and phosphorus (P)) measured in insoluble fraction from tissue samples of *Diopatra neapolitana* collected among Autumn of 2018, Winter, Spring and Summer of 2019 in sampling sites. Initials CD, M, SJ, T and CN stand for Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova.

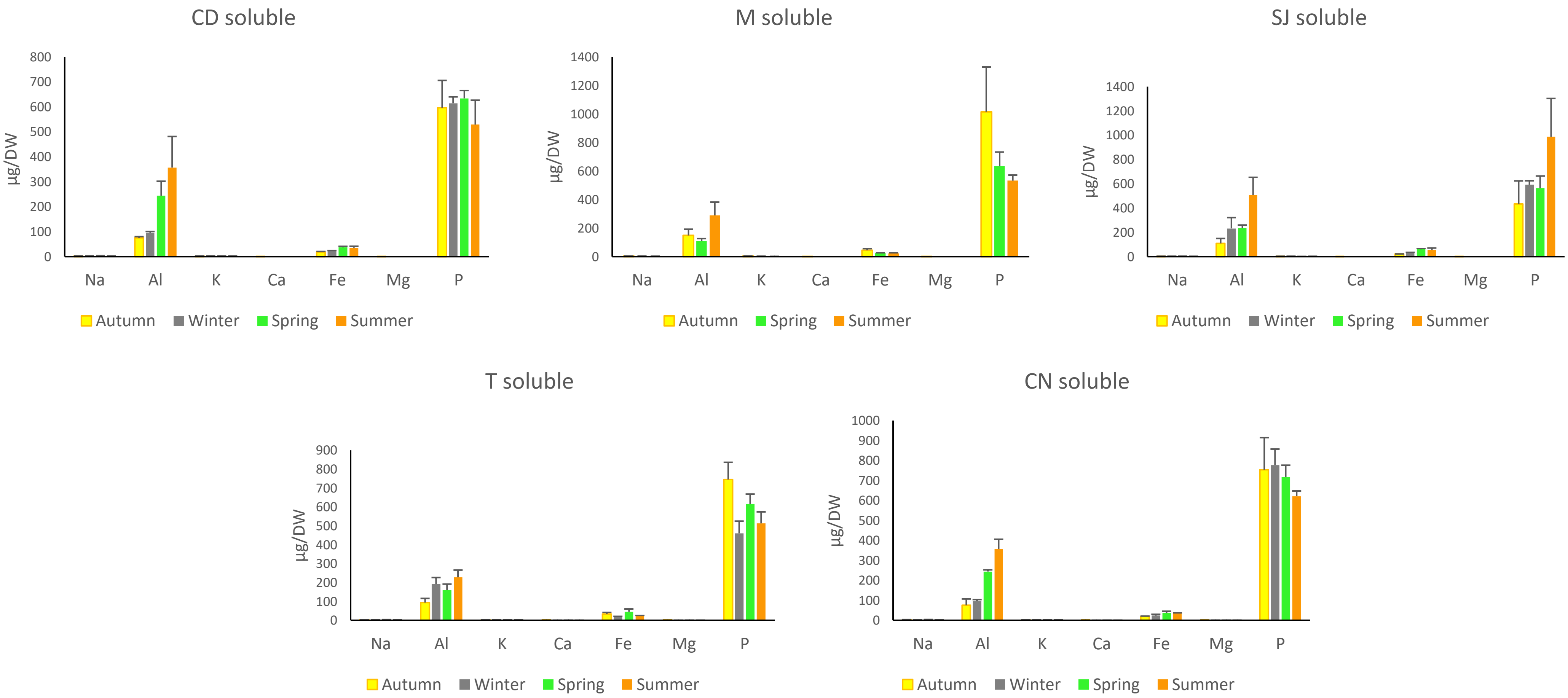

Figure S2. 3 Major elements bioaccumulation (aluminium (Al), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na) and phosphorus (P)) measured in soluble fraction of tissue samples from *Diopatra neapolitana* collected among Autumn of 2018, Winter, Spring and Summer of 2019 in sampling sites. Initials CD, M, SJ, T and CN stand for Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova.

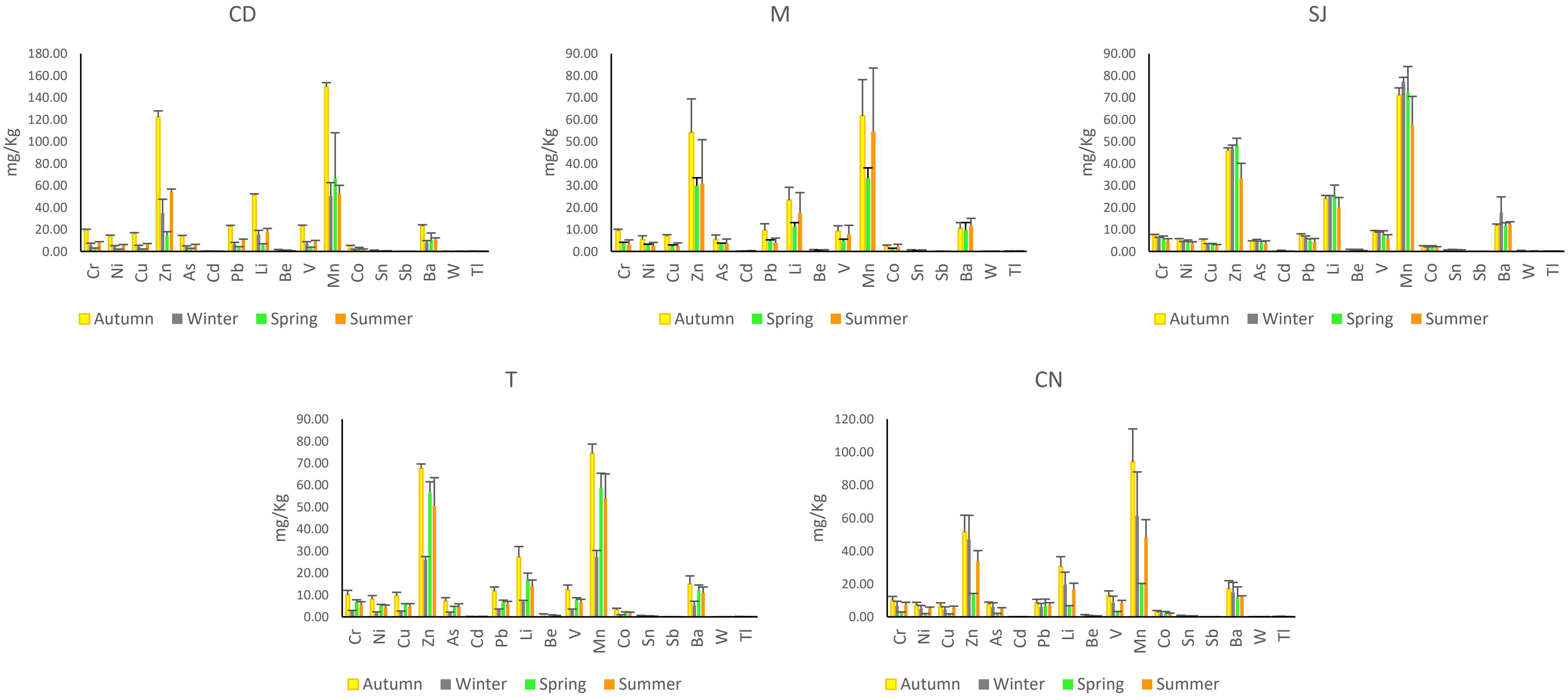

Figure S2. 4 Trace elements concentration (arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lithium (Li), manganese (Mn), nickel (Ni), lead (Pb), antimony (Sb), tin (Sn), thallium (Tl), vanadium (V) and tungsten (W)) measured in sediments collected among Autumn of 2018, Winter, Spring and Summer of 2019 in sampling sites. Initials CD, M, SJ, T and CN stand for Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova.

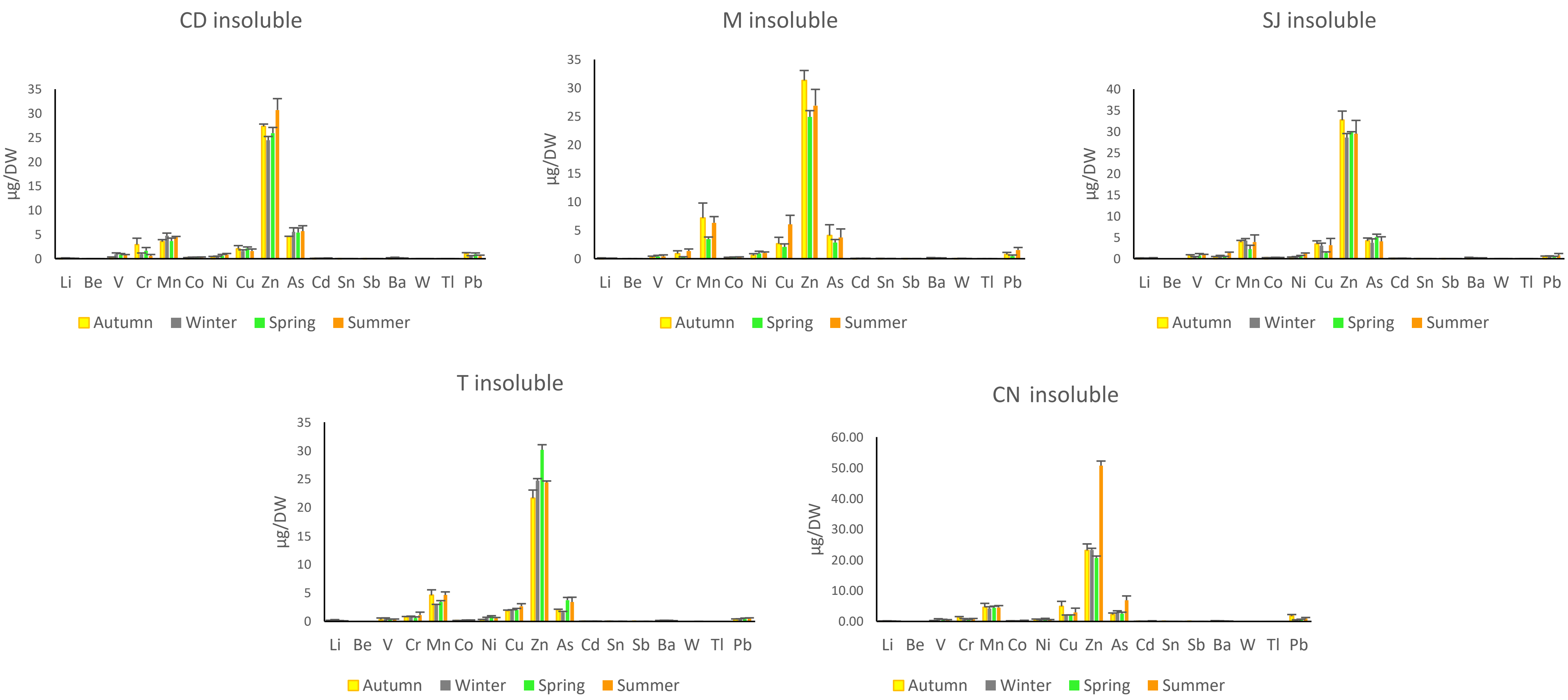

Figure S2. 5 Trace elements bioaccumulation (arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lithium (Li), manganese (Mn), nickel (Ni), lead (Pb), antimony (Sb), tin (Sn), thallium (Tl), vanadium (V) and tungsten (W)) measured in insoluble fraction of tissue samples from *Diopatra neapolitana* collected along Autumn of 2018, Winter, Spring and Summer of 2019 in sampling sites. Initials CD, M, SJ, T and CN stand for Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova.

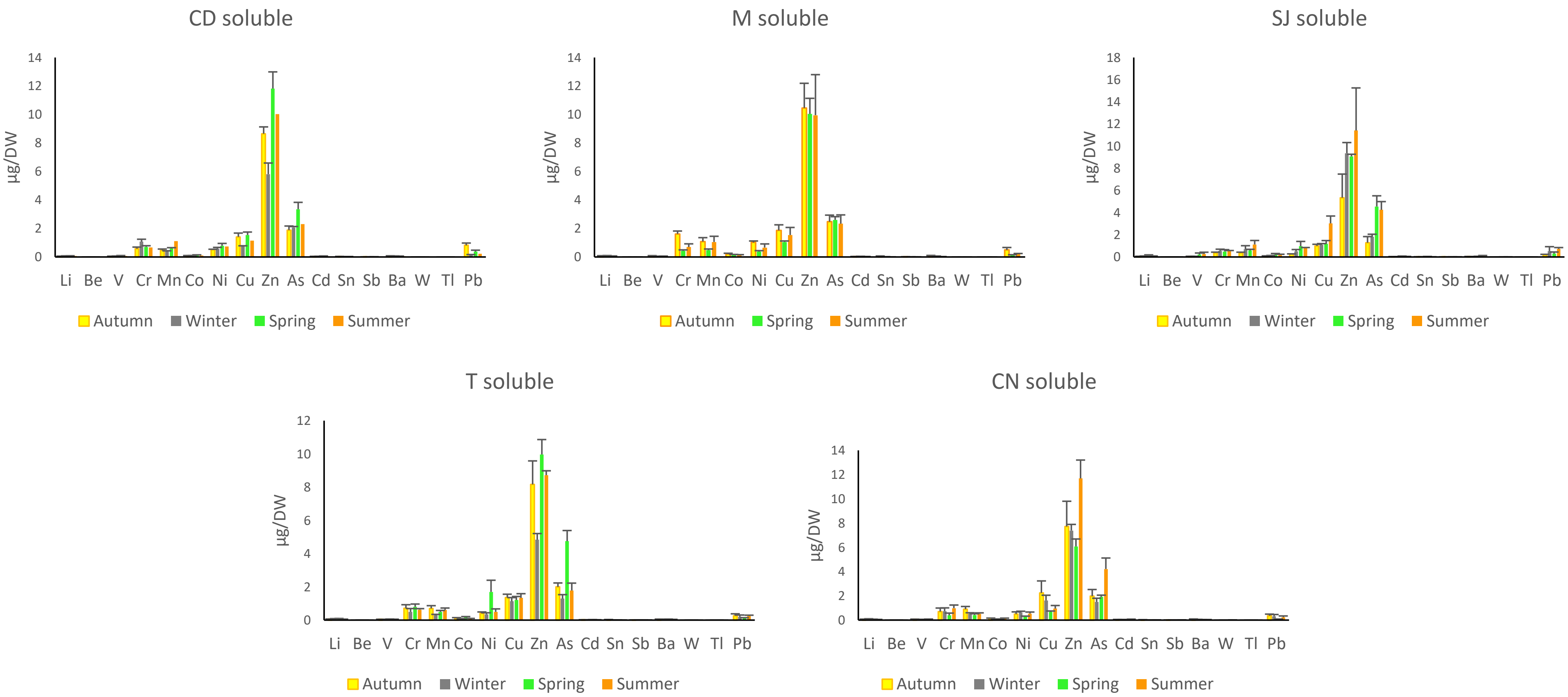

Figure S2. 6 Trace elements bioaccumulation (arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lithium (Li), manganese (Mn), nickel (Ni), lead (Pb), antimony (Sb), tin (Sn), thallium (Tl), vanadium (V) and tungsten (W)) measured in soluble fraction of tissue samples from *Diopatra neapolitana* collected along Autumn of 2018, Winter, Spring and Summer of 2019 in sampling sites. Initials CD, M, SJ, T and CN stand for Cale do Ouro, Murtosa, São Jacinto, Torreira and Costa Nova.

Chapter III- Supplemental files

Supplementary Table 1 Statistical analysis of the biochemical parameters measured in *Diopatra neapolitana* during Autumn of 2018, Winter, Spring and Summer of 2019 in CD: Cale do Ouro, M: Murtosa, SJ: São Jacinto, T: Torreira and CN: Costa Nova. Biochemical parameters measured were protein content (PROT), glycogen (GLY), electron transport system (ETS), catalase (CAT), superoxide dismutase (SOD), glutathione S- transferases (GSTs), non-protein thiols (NPT), lipid peroxidation (LPO) and protein carbonylation (ProC). Letters a, b, c and d means that exists significant differences between sites. Values are means of 3 replicates ± standard error.

Season	Site	PROT	GLY	ETS	CAT	SOD	GSTs	NPT	LPO	ProC
Autumn	CD	128.09±3.98[a]	14.09±0.69[a]	46.76±6.64[a]	17.71±1.04[a]	31.90±1.44[a]	0.03±0.01[a,b]	0.47±0.03[a]	12.45±1.80[a]	2.98±0.10[a]
	M	126.74±3.98[a]	22.93±0.78[b]	63.52±10.63[a]	18.03±1.89[a,b]	44.39±3.31[b]	0.02±0.01[a]	0.74±0.05[a]	12.81±2.45[a]	3.94±0.17[b]
	SJ	141.07±3.79[a,b]	19.17±1.33[c]	60.37±11.41[a]	19.76±2.03[a,c]	35.98±0.84[c]	0.03±0.01[a,b]	0.66±0.06[a]	8.89±0.64[a]	3.86±0.25[b]
	T	108.36±4.46[b,c]	19.75±1.35[b]	59.06±7.52[a]	22.28±1.14[b,c]	32.18±2.32[a,c]	0.03±0.01[b]	0.55±0.09[a,b]	9.53±1.07[a]	3.91±0.30[b]
	CN	112.70±3.29[c]	12.63±1.23[a]	55.41±6.66[a]	15.75±0.74[a]	25.12±0.91[d]	0.03±0.01[a,b]	0.73±0.07[b]	53.46±4.64[b]	4.75±0.21[c]
Winter	CD	120.90±9.32[a]	17.85±1.62[a]	81.90±10.41[a]	17.43±1.07[a]	59.82±4.91[a]	0.05±0.08[a]	0.70±0.11[a]	34.61±5.15[a]	3.17±0.24[a]
	SJ	133.40±7.04[a]	16.67±0.91[a]	100.26±18.02[a,b]	19.67±0.77[a]	61.68±3.99[a]	0.03±0.01[a]	0.80±0.06[a]	88.25±2.36[b]	3.81±0.18[b]
	T	126.48±8.64[a]	15.52±1.01[a]	122.63±12.13[b]	17.17±1.32[a]	60.84±3.30[a]	0.04±0.01[b]	0.65±0.04[a]	38.61±4.60[a]	2.93±0.15[a]
	CN	152.75±16.75[a]	15.11±0.48[a]	71.27±10.94[a]	18.13±0.84[a]	63.08±2.16[a]	0.03±0.01[a,b]	0.76±0.05[a]	55.32±3.32[c]	3.06±0.19[a]
Spring	CD	120.97±6.26[a]	5.86±0.54[a]	57.75±6.14[a]	27.48±2.55[a,b,c]	44.94±3.00[a]	0.03±0.1[a]	1.45±0.12[a]	16.54±2.19[a]	3.24±0.17[a,b]
	M	126.82±3.75[a]	6.98±0.60[a]	87.60±11.17[b]	33.09±1.70[b]	45.33±2.48[a]	0.04±0.03[a]	0.88±0.05[b]	15.78±3.29[a]	3.27±0.13[a]
	SJ	151.99±5.26[b]	5.37±0.36[a]	57.75±6.44[a]	23.61±1.09[c]	45.47±1.75[a]	0.02±0.01[a]	0.96±0.14[b]	21.18±1.97[a]	2.89±0.07[b]
	T	132.46±4.71[a]	5.99±0.62[a]	69.32±7.40[a,b]	28.28±2.21[a,c]	46.83±3.45[a]	0.03±0.01[a]	0.93±0.11[b]	17.48±2.52[a]	2.90±0.09[b]
	CN	123.22±5.84[a]	12.73±0.97[b]	54.88±6.01[a]	23.36±2.68[a,c]	51.10±1.98[a]	0.03±0.01[a]	0.58±0.04[c]	36.69±2.84[b]	3.63±0.19[a]
Summer	CD	123.58±3.47[a]	12.35±1.12[a]	120.41±11.00[a]	26.43±1.98[a]	43.46±2.02[a]	0.09±0.01[a]	1.00±0.12[a,b]	13.69±3.64[a]	3.16±0.20[a]
	M	133.07±8.34[a]	14.20±0.76[a]	86.93±12.72[b]	27.84±1.95[a,b]	39.64±1.15[a]	0.08±0.03[a]	0.94±0.11[a,b]	33.96±8.24[b]	3.15±0.12[a]
	SJ	125.70±2.56[a]	12.55±0.96[a]	94.56±12.75[a,b]	30.85±0.77[b]	39.12±1.39[a]	0.09±0.02[a]	0.73±0.08[a]	6.00±0.52[c]	3.02±0.13[a]
	T	125.57±5.92[a]	12.74±0.36[a]	92.19±7.14[b]	27.64±1.24[a]	40.78±1.35[a]	0.12±0.03[b]	0.83±0.05[a,b]	15.15±1.91[a,b]	3.47±0.17[a]
	CN	123.822±3.89[a]	14.75±1.01[a]	90.89±13.75[a,b]	25.06±1.84[a]	54.90±1.40[b]	0.08±0.02[a]	1.01±0.09[b]	15.08±1.79[a]	3.33±0.22[a]