UNIVERDIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Pesticides and polychlorinated biphenyls (PCBs) contamination and ecotoxicity in estuarine biota

João Pedro Pereira Azedo Cardoso

Mestrado em Ciências do Mar

Dissertação orientado por

Doutor Bernardo Afonso de Aranha Alhandra Duarte
Doutora Carla Maria Ferreira Mesquita Palma

2022

Pesticides and polychlorinated biphenyls (PCBs) contamination and ecotoxicity in estuarine biota

Agradecimentos

Primeiramente gostaria de agradecer aos meus dois coordenadores: Doutor Bernardo Duarte e Doutora Carla Palma pelo continuo suporte e orientação desta tese de mestrado.

Gostaria de agradecer à fundação FCT através dos projetos MARE (UIDB/04292/2020) e OPTOX (PTDC/CTA-AMB/30056/2017). Gostaria igualmente de agradecer ao Instituto Hidrográfico por me ter acolhido no meu estágio.

Agradeço aos grupos de laboratório, especialmente aos responsáveis que supervisionaram o meu trabalho e me guiaram no meu trabalho laboratorial: Engenheira Paula Santos, no laboratório de orgânica da Divisão de Química e Poluição do Meio Marinho do Instituto Hidrográfico, aos Investigadores Eduardo Feijão e Ricardo Carvalho no Laboratório de Biomonitorização e Ecotoxicologia (BIOTOX) da Faculdade de Ciências da Universidade de Lisboa.

Tenho de prestar a maior gratidão aos meus pais, Fátima Pereira e João Cardoso, por me terem suportado e acreditado em mim durantes estes anos todos, pois sem eles não tinha realizado este projeto.

Agradeço à minha namorada, Ana Sofia Oliveira, por toda a ajuda e carinho que me deu nesta etapa.

Agradeço a todos os meus amigos, por me terem sempre apoiado. Especialmente ao José Carlos pela sua disponibilidade para ajudar nos momentos mais difíceis.

Resumo

Poluentes são substâncias, misturas de substâncias, ou energias que são introduzidas no meio ambiente pelo ser humano, que apresentam a possibilidade de causar efeitos nefastos a sistemas ecológicos e/ou seres vivos. Os estuários são um exemplo de ambientes afetados por poluentes, visto que um terço da população mundial vive em zonas costeiras e estuarinas. Estes ecossistemas são bastantes importantes pelo seu alto teor de nutrientes, tornando-os assim em habitats com elevada importância ecológica com múltiplas espécies de diferentes reinos a ocupá-los, como por exemplo: peixes, molúsculos, crustáceos, aves migratórias, microalgas, fitoplâncton e entre outros. Também são ambientes com elevada importância turística e educação ambiental, sendo utilizados como laboratórios naturais.

Dentro dos poluentes orgânicos existentes, existe o grupo denominado como poluentes orgânicos persistentes, que são compostos cuja meia vida é longa, isto é, demoram consideravelmente muito tempo para o composto reduzir a sua concentração original para metade. Dois destes grupos de compostos que se encontram no ambiente estuarino, e podem afetar a ecologia do mesmo, são os pesticidas organoclorados e os bifenilos policlorados. Estes compostos antropogénicos entram nestes habitats através de esgotos ou de descargas ilegais.

Pesticidas são compostos orgânicos cujo propósito é de controlar, prevenir e eliminar qualquer tipo de praga, desde ervas, fungos, insetos e outras. Os pesticidas analisados neste trabalho foram os compostos DDT (diclorodifeniltricloroetano), pp' DDT e op'DDE e os compostos DDE (diclorodifenildicloroetileno), pp' DDE e op' DDE.

PCBs são compostos orgânicos que abrangem 209 moléculas contendo átomos de cloro, cujo nome e propriedades variam de acordo com a posição relativa dos átomos de cloro. São utilizados especialmente na indústria de pigmentos, tintas, eletrónicos, papel e cartão. Os PCBs analisados neste trabalho foram PCB 28, PCB 52, PCB 101, PCB 105, PCB 118, PCB 138, PCB 153, PCB 156 e PCB 180.

Estes compostos, devido ao seu elevado nível de toxicidade, foram proibidos em múltiplos países, porém, devido à sua persistência no ambiente, atualmente continuam a ser encontrados no mesmo, afetando as espécies que o habitam. Devido às capacidades lipofílicas destes compostos acabam por ter a concentração magnificada, agravando os seus efeitos tóxicos, e acabam por se encontrar nas cadeias tróficas dos ecossistemas.

Este trabalho teve como objetivo avaliar a concentração de pesticidas e PCBs em ambiente estuarino e os seus efeitos num modelo-espécie aquática.

Escolheu-se o estuário do Tejo, mais especificamente as zonas de Alcochete, Rosário e Seixal. Nestes estuários foram amostradas múltiplas réplicas de duas matrizes diferentes: sedimentos presentes nos sapais dos estuários e amostras vegetais de uma espécie de planta halófita, a *Spartina maritima*. As amostras vegetais foram divididas em parte subterrânea e aérea. Após o procedimento de amostragem foram analisados a granulometria e o carbono orgânico total dos sedimentos, e extraídos os compostos pesticidas e os bifenilos policlorados destas duas matrizes. Nos sedimentos recorreu-se ao processo de ASE e nas plantas foram utilizados QuEChERS. Seguidamente os extratos foram analisados através da técnica de cromatografia gasosa com um detetor de captura de eletrões (GC-ECD).

Foi possível observar que os sedimentos do Rosário apresentavam uma maior concentração em pesticidas em relação aos restantes estuários, devido à sua atividade agrícola, e o Seixal, por ser uma zona com maior atividade industrial, apresentava maiores concentrações de PCBs. Através da comparação com guias de qualidade ambiental, foi possível observar que os três sapais apresentavam contaminação por PCB 101, 118, 153, 180 e compostos PCBs. Foi igualmente possível observar que o Rosário apresentava poluição por pp' DDE e de compostos de DDE, tal

como o Seixal, de compostos DDD. O Seixal ainda apresentava poluição de PCB 28 e PCB 52. As concentrações dos compostos analisados, obtidas neste trabalho, foram igualmente comparadas com outros trabalhos que estudaram a concentração destes compostos em outros estuários portugueses, tendo-se observado que o Estuário do Tejo apresentava na sua maioria concentrações mais elevadas nestes poluentes. Este facto poderá estar relacionado com a sua elevada população e consequentemente elevada atividade, quer agrícola quer industrial.

Na planta verificou-se que a parte subterrânea apresentava maiores concentrações de poluentes do que a parte subaérea, o que significa que o transporte ocorrido entre as duas partes para estes compostos é mínimo.

De modo a observar os efeitos tóxicos a diatomácea modelo *Phaeodactylum tricornutum* foi exposta a diferentes concentrações de PCB 153 e foram analisados vários biomarcadores, biofísicos e bioquímicos das células-alvo.

A importância deste estudo reside no facto que o fitoplâncton, como é o caso da diatomácea testada, contribui com mais de cinquenta por cento do oxigênio disponível na atmosfera terrestre, assim como são a base da cadeia trófica de ambientes marinhos onde se inserem. Assim ao se expor estas espécies a compostos com propriedades tóxicas, lipofílicas, estaremos a prejudicar o ambiente em que vivem, afetando a ecologia dos ambientes em que estes se apresentam.

Os meios de cultura de *P. tricornutum* foram feitos em condições controladas durante quatro dias, sendo os dois primeiros dias para as células atingirem o seu pico de crescimento. De seguida, a espécie foi exposta ao PCB 153 a diferentes concentrações 0 µg/L, 1 µg/L, 3 µg/L e 6 µg/L. Esta exposição decorreu nos restantes dois dias. Observou-se diariamente o crescimento celular, de modo a perceber se a variação diária era influenciada pela exposição ao composto. No último dia a eficiência fotoquímica das espécies for analisada, de modo a determinar se a exposição ao PCB 153 afetou os fatores que influenciam diretamente a fotossíntese da espécie em causa. Seguidamente foram analisados diversos parâmetros, como a captação do poluente, através da extração com QuEChERS e análise com GC-ECD. Foram observados os níveis de pigmentos, a peroxidação lipídica e atividade enzimática anti oxidativa analisados por espetrofotometria.

Neste trabalho foi possível observar uma captação substancial do poluente PCB 153 pela espécie *P. tricornutum*. Esta captação está ligada ao facto de que, uma vez o composto PCB 153 é lipofílico, possui uma maior capacidade de ser captado pela membrana lipofílica das células, podendo ser magnificada e a longo prazo intensificar os efeitos tóxicos provocados nesta. Foi igualmente possível observar uma inibição do crescimento celular nos parâmetros analisados. Esta foi justificada por ser um efeito da inibição dos vários biomarcadores que representam a eficiência fotossintética, que foi ligado por sua vez ao efeito de stress oxidativo realizado pela interação da célula com o PCB 153. Tais efeitos foram, por exemplo: o fecho dos centros de reação; uma inibição no transporte de eletrões; e danos ao sistema funcional responsável pela fotossíntese. Como modo de resposta a estes efeitos tóxicos, as células incrementaram a eficiência fotoquímica, como a transferência de energia do fotossistema I. Nas células cujo centro de reação esteja fechado, esta energia é transportada para um centro de reação aberto do fotossistema II. Além disso, incrementaram também a eficiência do transporte do eletrão da molécula PQH_2 para o fotossistema I. Porém a célula teve que recorrer a mecanismos adicionais como o aumento de alguns pigmentos, como a clorofila *a*, clorofila *c* e fucoxantina que permitem à célula aumentar a sua capacidade de capturar fotões para o processo fotossintético. Verificou-se ainda um aumento de pigmentos fotoprotetores que evitam processos de inibição da atividade fotossintética, e um aumento da atividade de várias enzimas antioxidantes, de forma a combater o stress oxidativo.

Palavras-chave

Contaminantes orgânicos; PCBs; Pesticidas; Ecotoxicologia; Química Ambiental

Abstract

This project reports the analysis of organochloride pesticides and polychlorinated biphenyls (PCBs) compounds in a marsh environment, to observe its toxicological effects on a marine model organism.

To achieve this goal, it was quantified the concentration of organocloride pesticides and PCBs in sediments and plants, with origin in Alcochete, Rosário in Seixal estuaries. In this case, we chose to study *Spartina maritima*. To extract the pollutants, it was used the ASE and QuEChERS techniques in sediments and plants samples, respectively, and the GC-ECD technique to analyze its content.

With this experiment, it was possible to conclude that pesticide compounds were more predominant in Rosário, while PCBs compounds were more predominant in Seixal, derived from the activities that occurred in the vicinity of those sites. Alcochete was the less polluted site that was analysed in this work. When comparing the results obtained in this work with other concentrations from the same compounds in other Portuguese estuaries, it was possible to observe that the Tagus estuary presented a higher level of these contaminants. This is due to the higher population and activity that occurs in this zone, in comparison to other Portuguese estuaries. When studying the compounds' concentration on plants, it was possible to observe a higher concentration on the rhizosediments.

To test the toxicity of these compounds we exposed PCB 153 at 1 µg/L, 3 µg/L and 6 µg/L to the diatom model *Phaeodactylum tricornutum*. Afterwards, we analysed the biomarkers growth rate, photochemistry, pigment, fatty acids, lipid peroxidation and antioxidant enzyme activity of the cell.

It was possible to observe that, when exposed to the compound, the cells suffered from growth inhibition, oxidative stress, and photosynthesis inhibition, such as the closure of the reaction's centres and damage in the photosystem. This occured even though they presented counter mechanisms to the toxic effects, such as the incrementation of photosynthetic effects, such as the grouping probability, increase of efficiency of the electron transport from PQH_2 to PSI, and biochemical such as the incrementation of photoprotective pigments and pigments which increase the ability to trap photons for the reductions that happen in the photosynthesis and increases the activity of antioxidant enzymes.

Keywords

Organic contaminants; PCBs; Pesticides; Ecotoxicology; Environmental Chemistry

Index

Figure Index

Table Index

Symbology

δRo- Efficiency of the transfer of an electron from PQH_2 to final PSI acceptors
∑- Summatory
ABS/CS- Absorbed energy flux per cross-section
APx- Ascorbate peroxidase
Area- Size of the oxidized quinone pool
ASE- Accelerated solvent extraction
BAC- Background Assessment concentration
BC- Background concentration
CAT- Catalase
Chl *a*- Chlorophyll *a*
Chl *c*- Chlorophyll *c*
Chl a/c- Ration between the concentration chlorophyll a and chlorophyll c
d- Doubling time
DDE- Dichlorodiphenyldichloroethylene
DDT- Dichlorodiphenyltrichloroethane
DE- Diatomaceous earth
DES- De-epoxidation state
DI/CS- Dissipated energy per cross-section
DNA- deoxyribonucleic acid
EAC- Environmental assessment criteria
ET/CS- Electron transport energy flux per cross-section
ERL- Effects range low
FCP- Fucoxanthin-chlorophyll protein
GC-ECD- Gas chromatography coupled with electron captor detector
GP- Grouping probability
ISO- International Organization for Standardization
LC- Lower concentration
LOI- Loss of ignition
M- Divisions per day
Mo- The net rate of PS II RC closure
n- number of samples
N- Reaction centre turnover rate
NADP- Nicotinamide adenine dinucleotide phosphate
OCPs- Organochloride pesticides
OSPAR- Convention for the protection of the marine environment of the North-East Atlantic
PAM- Pulse Amplitude Modulated
PCBs- Polychlorinated Biphenyls
PEC- Probable effect concentration
Pheo a- Pheophytin a
POPs- Persistent organic pollutants
PSI- Photosystem I
PSII- Photosystem II
QuEChERS- Quick, easy, cheap, effective, rugged and safe
ROS- Reactive oxygen species
RC- Reaction centres
RC/CS- Number of available reaction centres per cross-section

RC/ABS- Reaction centre II density within the antenna chlorophyll bed of PSII
RE_0/RC- Electron transport from PQH_2 to the reduction of PSI and electron acceptors
RNA- Ribonucleic acid
SFA- Saturated fatty acids
SFI- Structure functional index for photosynthesis
SFI (NPQ)- Non-photochemical quenching or dissipation structure functional
SGR- Specific growth rate
Sm- Energy needed to close all reaction centres
SOD- Superoxide dismutase
TBARS- Thiobarbituric acid reactive substances
TEC- Threshold effect concentration
TOC- Total organic carbon
TR/CS- Trapped energy flux per energy cross-sectio

1. General Introduction

1.1. Pollutants and their physical-chemical properties

Pollutants are compounds or energies that are introduced by the human being into the environment, causing nefarious effects on living and/or ecological systems [1]. After being introduced into an aquatic biome, these pollutants suffer multiple physicochemical and biological processes such as evaporation, emulsion, dissolution, biodegradation/biotransformation and eventually sedimentation in the estuarine bottom and/or margin's sediments, the major pollutant sink [2].

Organochloride pesticides (OCPs) and polychlorinated biphenyls (PCBs) compounds are a type of pollutant denominated persistent organic pollutants (POPs). They are persistent in the environment due to their long half-life periods (the time it takes for a certain amount of a compound to be reduced by half). It's important to refer that these organic contaminants do not have a natural origin, and their presence in the environment results from anthropogenic activity [3].

There is a lack of information regarding its accumulation in one of the major estuarine sinks, the salt marshes. This fact reinforced the need to evaluate its accumulation profiles in these key habitats, as well as the impacts on estuarine primary producers such as phytoplankton.

1.2. Organochloride pesticides

Pesticides are defined as substances, or a mixture of substances, that have the purpose to prevent, control and eliminate any type of plague. In other words, it is a compound that is biologically active and therefore interferes with the well-being of the living species [4]. Since several species are considered plagues to agricultural activity, different types of pesticides have been produced to target this wide array of species, such as insecticides, herbicides, fungicides, and many others [4].

Pesticides have been used for as long as agriculture has existed [5]. But it was only a few years ago, after the Second World War, that the exploration of these substances suffered a boost in terms of development and application. This led to the production of several artificial pesticides, like dichlorodiphenyltrichloroethane (DDT) and other organochloride compounds. Previously to this boost in pesticide development, natural compounds were used (such as arsenic, sulphur, nicotine and a few more) to fight the spreading and impact of agricultural plagues [5, 6, 7].

The pesticides analysed in this work were DDT and its metabolite dichlorodiphenyldichloroethylene (DDE). DDT is one of the best-known pesticides and the most used during the 1950s. Mainly, it was used to control specimens that transported malaria and typhoid disease transmission vectors [8]. This pesticide and DDE have multiple enantiomers, such as the o,p'-DDT, p,p'-DDT, o,p'- DDE and p,p'- DDE [8].

DDT and its metabolites are known to cause hepatic tumours by activating the constitutive androstane receptor and inhibiting gap junctional intercellular communication. It can also cause oxidative stress [9]. These compounds are also known to cause tremors and convulsions [10]. Figure 1.1 presents the molecule formula of DDT and DDE.

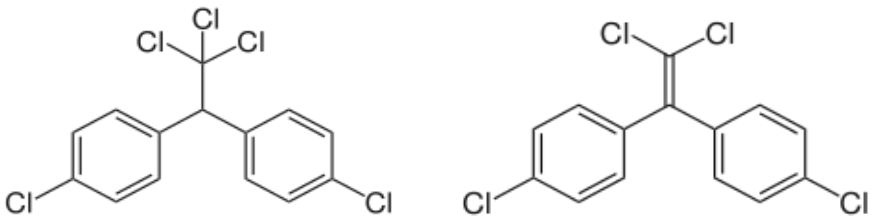


Figure 1.1- Molecule formula of DDT (left) and DDE (right)

1.3. PCBs

PCBs are a mixture of up to 209 molecules containing chlorine atoms. They vary the name according to the relative position of the chlorine atom in its chemical structure. A general structure of the PCB is presented in Figure 1.2 [3].

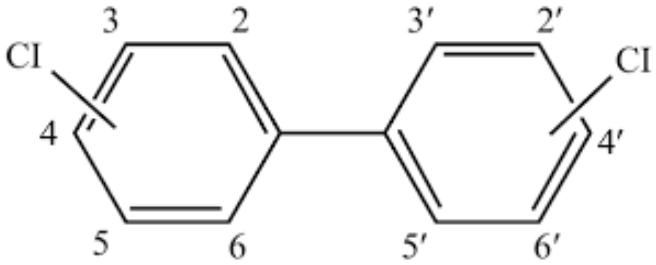


Figure 1.2 - PCB general chemical structure.

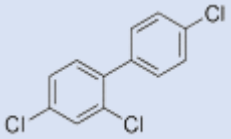
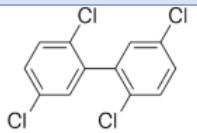
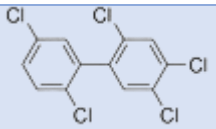
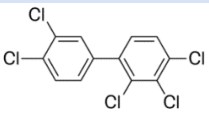
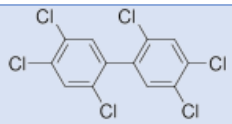
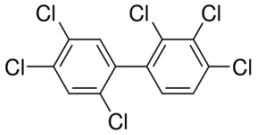
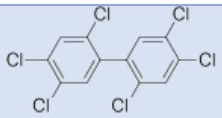
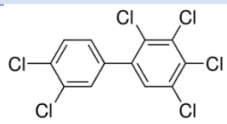
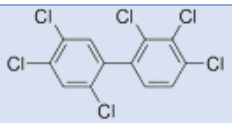
PCBs can have many functions depending on the number and position of chlorine atoms and their resulting chemical characteristics [11]. These compounds have applications in the industry of pigments, ink, electronics components, paper, and cardboard [3, 12]. For example, PCB 153 (or 2,2',4,4',5,5N'-Hexachlorobiphenyl) was used in PCB mixtures for the electric industry as a dielectric insulating fluid for transformers and capacitors [13].

Since PCBs are fat-soluble, they have a high bioaccumulation potential in living organisms [14]. PCBs can be divided into two different groups, dioxin-like, such as PCB 118, and non-dioxin-like. The latter have a bigger presence in the ecosystem but are less toxic. The dioxin-like have this denomination due to their toxic effects, such as the 2,3,7,8-tetrachlorodibenzo-p-dioxin (known as dioxin). PCBs tend to affect the human being by causing cognitive difficulties, cancer, asthma, arthritis, diabetes, liver disease and others [15]. In animal studies, they have been found to cause multiple toxic effects such as acute lethality, bodyweight loss, fatty liver, genotoxicity, neurotoxicity, and thyroid hormone-level alterations [14].

One of the effects caused by the PCBs is the inducing of DNA strand breaks and DNA repair. This is done through their metabolization, which will ultimately originate catechol. In this process, electrophilic arene oxide intermediates will be originated. These can bind to nucleophilic cellular macromolecules, such as DNA or RNA [14, 16]. Furthermore, arene oxide intermediates can be metabolized into methylsulfonyl metabolites. They tend to bind with proteins that will cause damage to the organism [14, 16]. It was also reported in Gosh (2010), that the presence of a chlorine atom is in the ortho position (2,2', 6 and 6' positions). This happens because it influences the ability to adapt co-planar conformation [11].

The PCBs that were studied in this work are presented in Table 1.1, with their respective molecular formula.

Table 1.1- Studied PCBs with corresponding molecular structure and formula.

Compound name	Molecular Structure	Molecule Formula
PCB 28		$C_{12}H_7Cl_3$
PCB 52		$C_{12}H_6Cl_4$
PCB 101		$C_{12}H_5Cl_5$
PCB 105		$C_{12}H_5Cl_5$
PCB 118		$C_{12}H_5Cl_5$
PCB 138		$C_{12}H_4Cl_6$
PCB 153		$C_{12}H_4Cl_6$
PCB 156		$C_{12}H_4Cl_6$
PCB 180		$C_{12}H_3Cl_7$

1.2. The objective of the study

The present work aimed to study the contamination of organochloride pesticides and polychlorinated biphenyls (PCBs) in three salt marshes of the Tagus Estuary, and the ecotoxicological impact of a PCB and its accumulation and phytoremediation in a marine model organism.

To reach this goal, the concentration of pesticides and PCBs in sediment and plant samples was first determined. Consecutively, and considering one of the most abundant PCBs found in the estuarine environment assessment, the impact of a specific PCB was studied using the model diatom *Phaeodactylum tricornutum* as the target species. More specifically, the variation of the

following biomarkers was studied: growth rate, photochemistry, pigment, fatty acids, lipid peroxidation and antioxidant enzyme activity in the diatom cells.

2. Pesticides and PCBs accumulation in *Spartina maritima* plants and rhizosediments in three salt marshes of the Tagus Estuary

2.1. Introduction

Bodies that are constituted by water, like seas, oceans, rivers and lakes, cover approximately 70% of the Earth's surface [17]. These water bodies are a crucial element for the existence of life on the planet Earth. Among the multiple ecosystem services provided by these water bodies, is the production of more than 50% of the world's oxygen by marine phytoplankton, macroalgae and marine plants (salt marshes and seagrass prairies) [18]. They also act as feeding grounds, habitats and shelter areas for many animal species, and sources of food, minerals, and energy, which in this case are mostly renewable [19, 20].

Estuaries are aquatic biomes located between the river and the ocean, composed mostly of brackish waters, due to the interaction between marine salt water and the riverine freshwater discharge [21]. These ecosystems are rich in nutrients arriving from the river basin, resulting in a highly productive area and a privileged habitat for many species of fishes, molluscs, crustaceans, and migratory birds. They are, therefore, attractive for human activities such as tourism and fishing, but also very important in terms of environmental education, acting as natural laboratories [22]. In floristic terms, these types of biomes are habitats for multiple species of macroalgae, seaweeds, plants and phytoplankton [23]. Nevertheless, and since a third of the world population inhabits coastal and estuarine areas, these ecosystems are very prone to anthropogenic pressures, namely pollution [24].

The Tagus estuary is the largest estuarine system in Portugal, harbouring also one of the largest wetland areas. On its north margin, it harbours the Portuguese capital, the Lisbon metropolitan area, a key factor for its social and economic development. On the south margin, this biome is divided into three sections: Tagus' estuary natural reserve, comprised by the Benavente and Alcochete municipalities; a second area comprising Esteiros da Moita and Baía de Sarilhos that developed in the locations of Moita, Alhos Vedros, Gaio-Rosário and others; and finally, the last sector comprising Esteiros de Seixal and Coina that bathes Corroios, Amora, Seixal and Coina [20].

This estuary has several pollution sources, from agricultural runoffs, which were the main responsible for pesticide pollution, to several chemical, petrochemical, metallurgic, cement and shipbuilding industries [25]. The industries are in their majority located in both margins of the Tagus estuary, the Vila Franca de Xira-Alverca and the Barreiro-Seixal industrial regions [26, 27, 28, 29].

Salt marshes are presented in estuarine and coastal ecosystems, being inhabited by vegetation species that are particularly tolerant to the salinity and submersion conditions present in these ecosystems [30]. This halophytic is adapted to resist/tolerate and complete its life cycle in ecosystems with salt concentrations above 200 mM [31-34]. They are also a highly resilient species, a characteristic that allowed them to develop a high tolerance to other environmental stressors, such as flooding or pollution [31-34]. Salt marshes are known to act as natural barriers against floods and coastal erosion and can dissipate waves energy reducing the risk of storm surges [35]. Although these barriers are not effective against extreme hydrodynamic features, their value as a natural barrier has been recognized [35].

The presence of halophytic vegetation on estuaries increases sediment deposition on the margins during high tide [35]. These sediments are usually constituted by fine grains, having a high degree of affinity to contaminants [36]. Nevertheless, salt marsh vegetation acts as a natural remediator of this anthropogenic contamination, trapping these potentially toxic compounds in the sediments and within their biomass [25, 37].

Spartina maritima, an example of this type of vegetation, is one of the most common halophyte species present in European and Portuguese transitional systems [38]. This species of halophyte populated the northern hemisphere, being native to the western and northern European coasts, as well as the western African coast. It is also possible to find it on the Atlantic coasts of Namibia and South Africa [38]. Additionally, this species is known to have a high remediator capacity due to their phytoremediation capacity (Redondo-Gómez, 2013) [39].

Since pesticides and PCBs are anthropogenic compounds, they find their way to these environments by discharges, accidents, effluents, landfills, incineration, combustion and others [3].

Nevertheless, these compounds present a high persistence in the environment, having a high potential for accumulation in natural sinks, namely in the estuarine compartment. [40]

According to Schafer *et al.* (2007), these pollutants are highly soluble in fats, oils, lipids and non-polar solvents, i.e., they have a high degree of lipophilicity, hence displaying a higher tendency to bioaccumulate in living organisms [41]. Since these contaminants are persistent in the environment, they tend to accumulate in aquatic species, influencing all the trophic chain and all its intervening [42].

To evaluate the environmental status concerning its contaminants pollution, several organizations, such as the United States Environmental Protection Agency (US EPA), the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) and the Wisconsin Department of Natural Resources, created different guidelines recommended for these pollutants in sediment matrix.

EPA recommends that these substances are present in values below the Effects Range Low (ERL) values. The ERL indicates the concentration below which toxic effects are scarcely observed or predicted [43].

OSPAR recommendations are based on the Background Assessment Concentration (BACs) i.e., the values in which the concentrations are near background/environmental levels or close to zero in the case of man-made substances. To calculate these values, a background concentration (BC) or lower concentration (LC) is chosen; and in Environmental Assessment Criteria (EACs), values in which no chronic effects are expected to occur in marine species [44].

According to Wisconsin Department of Natural Resources, contaminant thresholds are based on two parameters: threshold effect concentration (TEC) and probable effect concentration (PEC), concentration values above which the toxicity to benthic-dwelling organisms is unlikely or probable, respectively [45].

These values are presented in Table 2.1.

Table 2.1- Sediment Quality Guidelines (SQGs) values recommended by US EPA, OSPAR commission, and Wisconsin Department [43, 44, 45].

	US EPA (µg/kg)	**OSPAR (µg/kg)**			**Wisconsin Department (µg/kg)**	
	ERL (1)	BC/LC (2)	BAC (3)	EAC(4)	TEC (5)	PEC (6)
PCB 28	-	0.0/0.05	0.22	1.7	-	-
PCB 52	-	0.0/0.06	0.12	2.7	-	-
PCB 101	-	0.0/0.07	0.14	3	-	-
PCB 118	-	0.0/0.08	0.17	0.6	-	-
PCB 138	-	-	-	-	-	-
PCB 153	-	0.0/0.10	0.19	40	-	-
PCB 180	-	0.0/0.11	0.1	12	-	-
Σ PCBs	11.5	0.2	0.46	-	60	676
pp'- DDE	2.2	0.0/0.05	-	-	-	-
pp' DDT	-	-	-	-	-	-
Σ DDTs	1.58	-	-	-	4.2	63
Σ DDEs	-	-	-	-	3.2	31

(1) ERL means effects range low
(2) BC/LC means background/low concentrations
(3) BAC represents background assessment concentration
(4) EAC represents the environmental assessment criteria
(5) TEC means the threshold effect concentration
(6) PEC is the probable effect concentration

These values are important since they permit an evaluation of the results that are obtained in monitoring programs and others.

2.2. Materials and methods

The procedures used in this work to analyse the chemical and physical parameters of sediments' samples were accredited according to ISO 17025, which are the standards that the Instituto Hidrográfico follows for such analysis. It is also important to notice that all experiments were subject to quality control, mainly done in duplicates, blank samples, recovery tests, use of certified reference material and participation in interlaboratory tests.

2.2.1. Plant and sediment field sampling

Sediments and plant samples were collected in November 2020 at three salt marshes located in the Tagus estuary Alcochete salt marsh (38º 45.661' N, 8º 56.116' W), Rosário (38º 40.161' N, 9º 00.198' W) and Seixal (38º 38.313' N, 9º 07.191' W)), during low tide (Figure 2.1). *Spartina maritima* plants and sediments were sampled (n=5 per site). Since the sediment samples were collected around the plant's, they can be nominated as rhizosediments. These samples were collected (0-5 cm depth), with a stainless-steel scoop into a plastic box. Samples were brought back to the laboratory in refrigerated bags and stored at -20 ºC until further analysis.

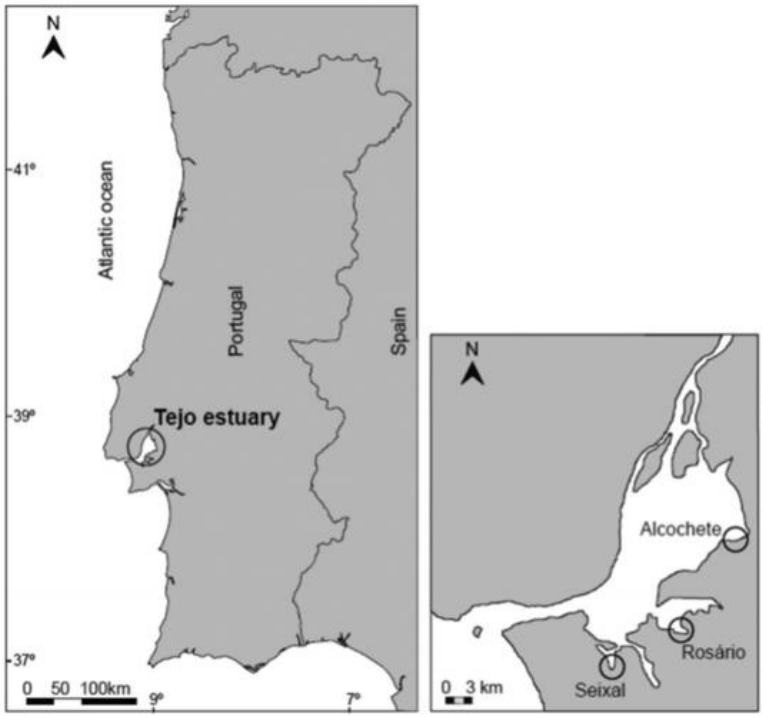


Figure 2.1- Map of the location of the Tagus Estuary, and the location of the sampling sites.

2.2.2. Sample pre-processing

After sampling, rhizosediments samples were divided into two fractions: one for the granulometry analysis and the other for the analysis of total organic carbon (TOC), humidity, organic matter, pesticides and PCBs concentrations.

This last fraction was fractionated with a sieve with a mesh of 2 mm. After this process, samples were frozen at -20 ºC in Petri plates and freeze-dried. In this procedure, approximately 5 µm Hg of pressure was applied, at -40 ºC, for up to 4 days, until samples were dried. In this methodology, a freeze dryer (Labconco LyphLock 1L) was used. Afterwards, the samples were milled using an agate Retsch RM 200 mill and Fritsch Pulverisette 2.

Plants' samples were washed to remove sediment particles, algae or other unwanted particles, divided into aboveground and belowground parts, and sliced in a Magic Bullet blender before the analysis of pesticides and PCBs concentrations.

2.2.3. Granulometry's analysis

The granulometry analysis process used in this experiment can be separated into two parts: sifting and the usage of the laser diffraction technique.

This first methodology followed the NT.LB.22 [46]. Sediment samples were submitted to an organic matter attack using hydrogen peroxide (H_2O_2).

Subsequently, the sample was heated in a water bath at 80 ºC, with gas extraction for five to nine days so that the attack on the organic matter is finished by evaporation of the excessive H_2O_2. It is important that the sample is agitated multiple times a day and that it does not dry. It is necessary to add water type III to ensure this. Then, the sample was cleaned in a vacuum, using porcelain containers, for the extraction of the salts dissolved in the previous step. This procedure was repeated five times [46].

Afterwards, the samples were fractionated in a sieve column (31.5 to 0.063 mm of mesh) [46].

For the fraction of the sample with granulometry inferior to 0.500 mm, we used the lased diffraction technique, and resorted to the MALVERN MASTERSIZER HYDRO 2000 G, as is described in the NT.LB.23 [47].

2.2.4. Total Organic Carbon

The total organic carbon (TOC) of the samples analysed in this work corresponds to the difference between the results of total carbon and total inorganic carbon. Total carbon was determined by converting it into carbon dioxide, through combustion, and total inorganic carbon by acidification of the sample and then purging of the CO_2, following the NT.LB.26 [48]. Both procedures were made and then quantified in an elemental analyser of carbon and nitrogen SKALAR Primacs SN100907. This was measured by non-dispersive infrared absorption spectrometry and the result was obtained in absorbance. The concentration of CO_2 was given directly.

2.2.5. Sediment water and loss on ignition

To study the sediment's water content, approximately 1 g of sample was submitted to a process of successive drying in a kiln at 105 ± 5 ºC, until its mass stayed constant. For these samples, two drying processes were required: the first one took 16 h and the second one took 1 h. After each drying process, the sample was weighted [49]. The sediment's water content was calculated with the following expression:

$$H = \frac{(mb\text{-}mc)}{(mb\text{-}ma)} \times f \quad (2.1)$$

With this process the dried matter could be calculated using the equation:

$$W_{dry} = \frac{(mc - ma)}{(mb - ma)} \times f \quad (2.2)$$

Where:

- H- humidity content

- W_{dry}- dry weight
- m_a- the weight of the Petri Plate
- m_b- the weight of the Petri Plate with the humid sample
- m_b- the weight of the Petri Plate with the dried sample
- f- a factor that depends on the unit it is pretended on the results: if it is in percentage, it is 100; if it is in g/kg, the factor is equal to 1000

Afterwards, approximately 1 g of dried samples of each sample were weighted in a crucible, for determination of the loss on ignition, often used as an estimate for the content of organic matter in the sample. In this process, an oven was used at 550 ºC for 2 h in the first ignition, while on the second ignition it was only used for an hour, as described by Heiri *et al*. (2001) [50]. The result was calculated from the following mathematical formula:

$$W_{LOI} = \frac{(md-mc)}{(md-ma)} \times 100\% \quad (2.4)$$

Where:

- w_{LOI}- the loss on ignition of the dry mass of a solid sample, in percentage (%);
- m_a- the mass of the empty crucible, in grams (g);
- m_d- the mass of the crucible containing the dried sample, in grams (g);
- m_c- the mass of the crucible containing the ignited sample, in grams (g);

2.2.6. Pesticides and PCBs extraction and quantification

PCBs and pesticides in sediments were extracted by resorting to the accelerated solvent extraction technique (ASE), using a Dionex Accelerated Solvent Extractor. Approximately 15 g of sediments were mixed with approximately 8-9 g of diatomaceous earth (DE), to fill the cell, as is required by U.S. EPA METHOD 3545 [51, 52, 53, 54]. The extraction operating condition is presented in Table 2.2.

Table 2.2- Conditions of the program used in the ASE technique.

Parameter	Experimental Condition
Solvent	n-hexane:acetone (50:50, v/v)
Pressure	2000 psi
Temperature	100 ºC
Number of cycles	2
Purge's time	180 *s*
Solvent volume	Half of the extraction cell's volume

After this process, some sulphur was removed from the samples by adding activated copper. The used amount depended on the deposition of the sulphur that is noticed on the copper.

The next step consisted of concentrating the samples in a Heidolph Laborota 4000 rotary evaporator. During this process, we ensured that the samples did not concentrate until dryness, since that could cause partial or total loss of the analytes. Afterwards, the samples were put at nitrogen flow until they were concentrated at approximately 2 mL.

Subsequently, the samples were purified using a column of silica gel and basic alumina, both deactivated at 5 % using milli-Q water. This column was made using n-hexane as a solvent, glass wool, 5 g of silica gel, 5 g of basic alumina, and approximately 1 cm thick of anhydrous sodium sulphate. The packing of the column was made carefully to prevent the formation of fissures and air bubbles in it, and we ensured that the column never dried. After the addition of the sample, the column was cleaned with two doses of 5 mL, each, of n-Hexane. After the second extractant, 25 mL of n-Hexane was added [55].

After this, the samples were concentrated in the rotatory evaporator as previously done. Then, the solution was transfered to tubes of 10 mL, and the necessary amount of isooctane was added to bring the volume to the triple of the concentrated extract. Afterwards, the samples were let at the nitrogen flow until 1 mL.

To extract pesticides and PCBs contained in the belowground and aboveground part of *Spartina maritima*'s samples, we used QuEChERS.

First, approximately 15 g of plant sample was transferred to a 50 *mL* tube. Subsequentially, we added 15 mL 1% acetic acid in acetonitrile. In this case, 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 1 g of monosodium citrate and 0.5 g of sodium citrate dibasic sesquihydrate were used. Then, it was shaken in the vortex for 1 minute and centrifuged at 1500 rpm for 1 min, as described by Lehotay (2007) [52].

Afterwards, 5 mL of supernatant was transferred to another 50 mL tube and a clean-up salt mixture was added. We used 150 mg of $MgSO_4$, so it could absorb the water remaning in the extract, 25 mg of primary-secondary amine (PSA), so it could eliminate the high concentrations of sugars, organic acids or fatty acids contained in the sample, and, because this sample presented high levels of pigments, 2.5 mg of graphitized carbon black (GCB). Then the tube was shaken at

the vortex for 30 s and centrifuged at 1500 rpm for 1 min [52]. Afterwards, the sample was concentrated until almost dry at a nitrogen flow. Finally, 1 mL of isooctane was added to the sample.

For both sediments and vegetal samples, before they were put in vials, 100 µL of internal standard, in this case PCB155 and PCB198, was added to the sediments extract and 25 µL to the plant extract. This procedure allows to facilitate the analysis of the peaks by gas-chromatography with electron capture detector (GC-ECD). Copper was also added to all samples and activated to guarantee that all the sulphur present in the sample does not interfere with the analysis. In this procedure five standard solutions were also prepared, each with a different known concentration, to make the calibration of the method [55].

We use the GC-ECD to quantify the content of the analytes of interest in these samples. In this project we used the Hewlett Packard 6890 Series GC gas chromatograph equipped with ^{63}Ni-electron capture detector (ECD) for sediments and Agilent 8890 GC System for plants extract content analysis, using a fused silica CP-Sil 8 CB column (60 m length, diameter 0.25 diameter, 0.1 µm film thickness) [55]. The operating conditions are presented in Table 2.3.

Table 2.3- Operating conditions of GC-ECD technique.

Component	Parameter	Condition
Syringe	Capacity	10 µL
Injector	Temperature	250 ºC
	Mode	Pulsed splitess
	Injected volume	2 µL
Furnace	Initial temperature	80 ºC (3 min)
	Heating ramp 1	30 ºC min^{-1} until 155 ºC
	Heating ramp 2	2 ºC until 188 ºC
	Heating ramp 3	3 ºC until 245 ºC
Carrier gas	Helium	20 mL min^{-1}
µECD Detector	Temperature	320 ºC
	Electron capture	µECD-Ni^{63}
Detector's Auxiliar gas	Argon/Methane	60 mL min^{-1}

The next step was to identify the peaks corresponding to each pesticide or PCB and quantify the concentration of the analyte. Table 2.4 presents the analyte with the corresponding retention time.

Table 2.4- Approximated time, in minutes, of the peak that corresponds with the determined analyte.

Analyte	Retention Time (min)
PCB 28	22.35
PCB 52	24.90
PCB 155	32.98
op' DDE	33.92
PCB 101	34.30
pp'- DDE	38.93
PCB 118	45.09
op' DDT	47.57
PCB 153	49.97
PCB 105	50.34
pp' DDT	53.87
PCB 138	54.35
PCB 156	60.82
PCB 180	62.69
PCB 198	66.00

To determine the concentration of a certain analyte, the following expression was used:

$$[X]=\frac{Y}{b} \quad (2.5)$$

Where:

- [X]- Concentration of X analyte presented in a certain sample (μg)
- b – Slope of the linear calibration used in this experimentation
- y – Instrumental sign (peak's height) expressed in *Hz*.

To convert the final result into µg.Kg^{-1}, we used to the following expression [56]:

$$[X]_{final}=\frac{Y}{b} \text{ x } \left(\frac{Vext*1000}{Vinj}\right) \text{ x } \left(\frac{1000}{m}\right) \text{ x } \frac{100}{Wdry} \text{ x } \frac{100}{RECX} \quad (2.6)$$

Where:

- $[X]_{final}$ – Final concentration of X analyte
- V_{ext} – Final volume of the organic extract (in this case, 1 *mL*)
- V_{inj} - Volume injected into the column, in this case, 2 μL
- mA - Initial mass of the sample
- W_{dry} – Dried weight of the sample
- REC_X – Recovery of the analyte X.

2.2.7. Quality control

To ensure quality control of pesticides and PCBs analysis in sediments and plants, a blank sample of the procedure was made, and a fortified blank sample and duplicate samples were prepared. For blank and fortified samples, only DE was added in the extraction cell, and in the

case of the fortified blank, 100 µL of fortified standard was added. Fortified samples were also made to test the quality of the experiment. Using this procedure, the recovery of the test could be calculated using the following expression:

$$REC_X\ (\%) = \frac{[X]_{BF}}{[X]t} \times 100 \qquad (2.7)$$

Where:

- RECx – Recovery of X analyte in the organic extract in an inert matrix
- $[X]_{BF}$ – Concentration of X analyte that was observed in the fortified blank; in the case of the plant sample, it would be the concentration of the fortified sample minus the concentration of the non-fortified sample.
- [X]t – Theoretical concentration of X analyte that was initially on the fortified solution added to the inert matrix

The criterion for acceptance of the blank samples was that their concentration should be inferior to the limit of detection (LD) of the method. In the case of the recovery tests, the criterion was that its value should be between 70 % and 130 %.

For the tests made in duplicate, the acceptance criterion considered was that the relative difference between the results should be inferior to 19,5%.

2.3. Statistical analysis of the results

To analyse the data of each of the attained variables, boxplots were employed using the ggplot-2 package in R-Studio (Version 4.1.2). In these graphs, the Kruskal-Wallis test with Bonferroni correction was applied as a means to compare the same variable among samples with different origins. In this work, statistical significance was considered at $p<0.05$.

2.4. Result's

2.4.1. Granulometry

The composition of the samples studied in this work is presented in Figure 2.2, representing each composition percentage. The standard deviation between the sites is also displayed, for each type of grain. The red boxplots (the left ones) represent Alcochete sample values, the green boxplot (the one at the center) is Rosário's result interval and the blue ones are Seixal (at the right).

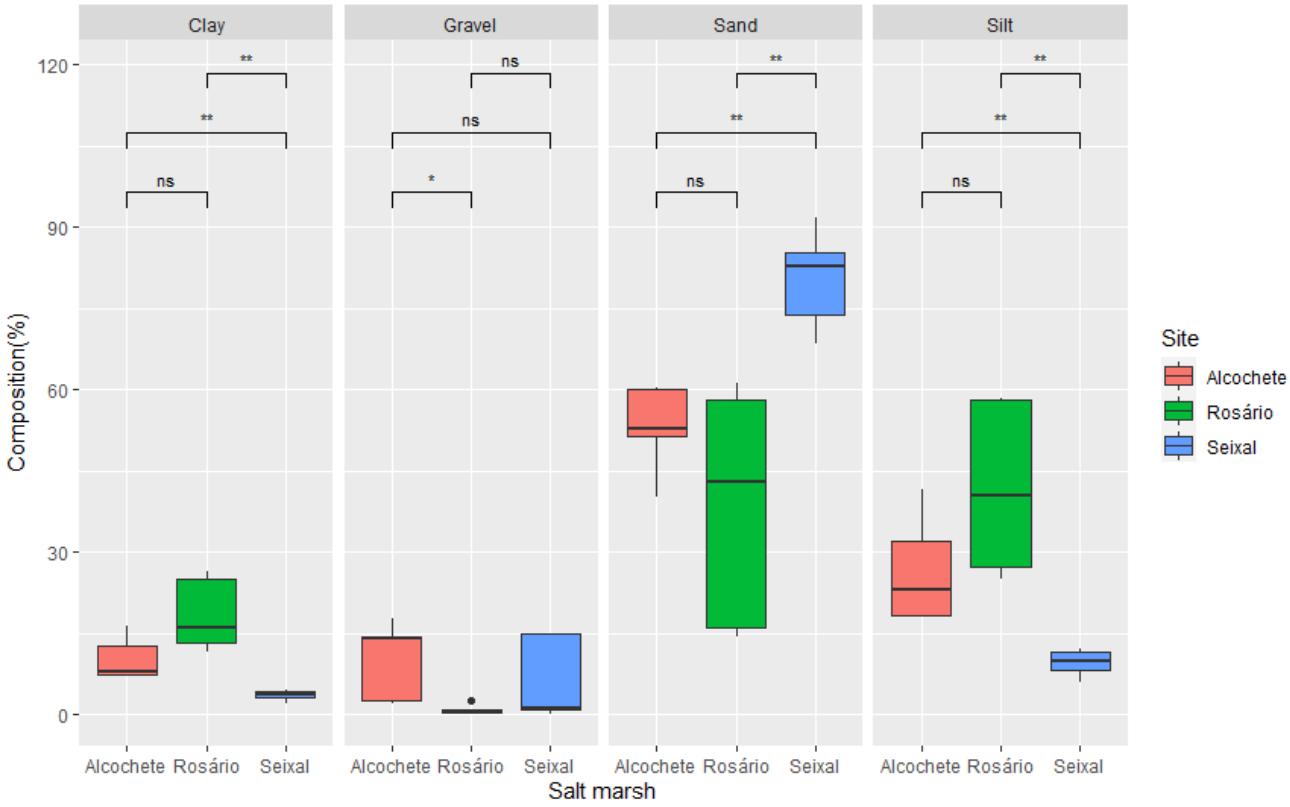


*Figure 2.2- Boxplot's representative of the results of granulometry of the sediments samples in Alcochete Rosário and Seixal sediments' samples. With the respective medium, acceptance intervals (25-75% range) and outliers. N=5. NS means non-significance and the asterisk represents statistically significant, with * representing p<0.05 and ** representing p<0.01.*

In this figure, it could be seen that there is a significantly lower clay and silt content, and an increase in sand content in Seixal sediments when compared to the other sites ($p<0.01$). This implies that there is a big difference in the composition of these types of grains between those sites.

The content of gravel presented in the sampled sediments originated from Alcochete showed a significantly higher percentage when compared to the gravel composition obtained in Rosário.

2.4.2. TOC and loss on ignition

In Figure 2.3, we present a boxplot of the the total organic carbon and loss on ignition (LOI). By analysing the results presented in this figure, we verify a significantly lower mass of the

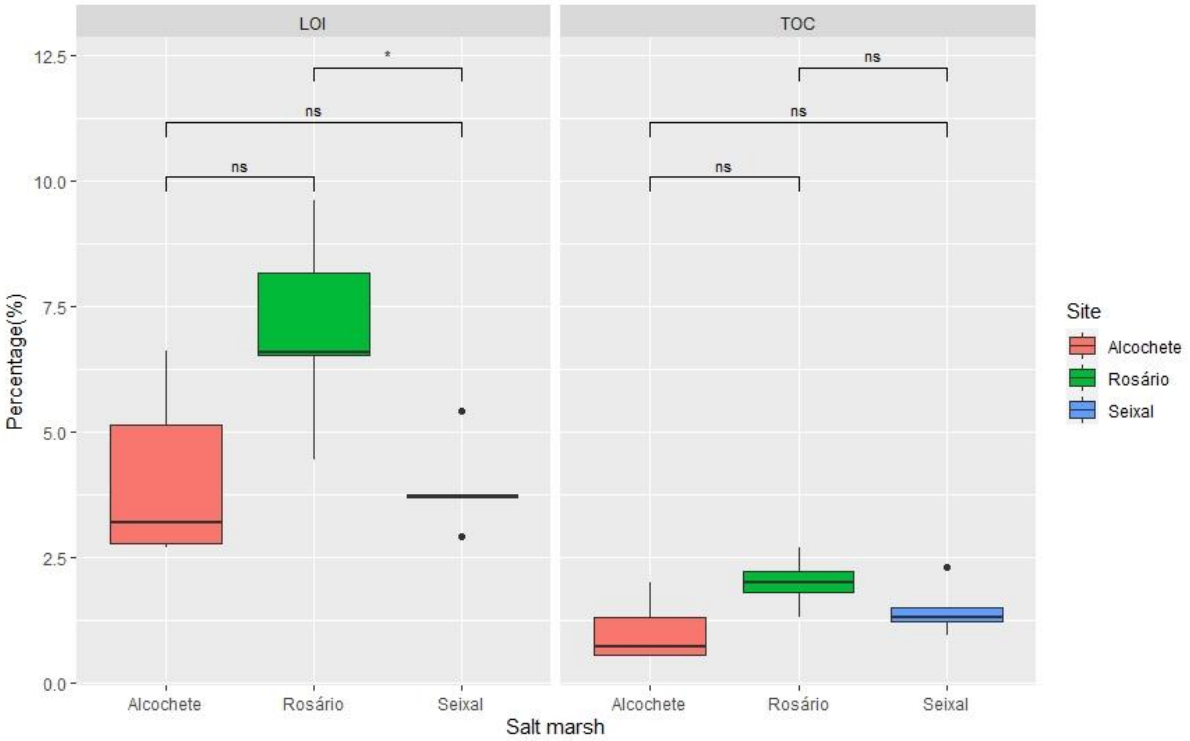


*Figure 2.3- Boxplot's representative of the results of loss of ignition (LOI) and TOC of the sediments samples in Alcochete, Rosário and Seixal. With the respective medium, acceptance intervals (25-75% range) and outliers. N=5. NS means non-significance and the asterisk represents statistically significant, with * representing p<0.05 and ** representing p<0.01.*

sediments lost by ignition from Seixal's sediments when compared to Rosário's LOI ($p<0.05$).

In this figure, it is also possible to notice that no statistical difference was noticeable between all the sites when compared to the total organic carbon present in the samples.

2.4.3. Sediments and Plants' pesticides and PCB content

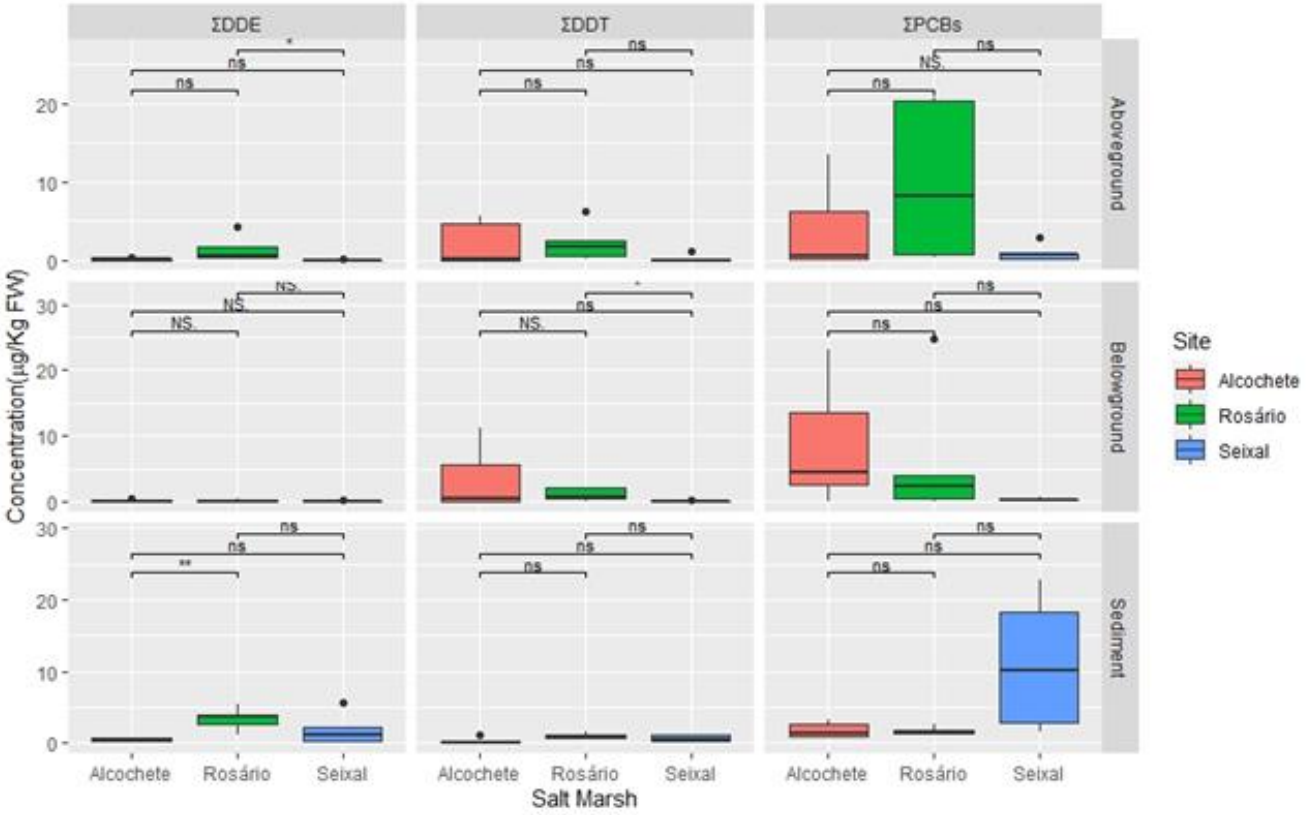


*Figure 2.4- Boxplot's representative of the results of the concentration of the ∑DDE, ∑DDT, ∑OCPs, and ∑PCBs presented in the plant (aboveground and belowground) and in sediments' samples sampled in Alcochete, Rosário and Seixal. With the respective medium, acceptance intervals (25-75% range) and outliers. N=5. NS means non-significance and the asterisk represents statistically significant, with * representing p<0.05 and ** representing p<0.01.*

Figure 2.4 presents the interval of results in form of boxplots, in µg/kg fresh weight (FW), obtained from the quantification of the pesticides and PCBs extracted from sediments and plants aboveground and belowground levels. Each one of these boxplots was made for each compound analysed and each type of sample, separately: ΣDDE (sum of op'DDE and pp'DDE's concentrations), ΣDDT (sum of op'DDT and pp'DDT concentrations) and ΣPCBs (sum of PCB 28, 52, 101, 105, 118, 138, 153, 156 and 180 concentration) compounds.

In Figure 2.4, we verify that, concerning the aboveground part of the plants, there was a significant higher ($p<0.05$) DDE compounds' content presented in the sediment sampled in Rosário when compared to the concentration of these compounds on Seixal's sediments.

Concerning the belowground plant parts, there is a significantly higher ($p<0.05$) DDT compounds concentration presented in Rosário's sediments when compared to the concentration of the same compounds in Seixal's sediments.

DDE compounds' concentrations on sediments were significantly higher ($p<0.01$) on Rosário than the Alcochete's DDE content presented in its sediments.

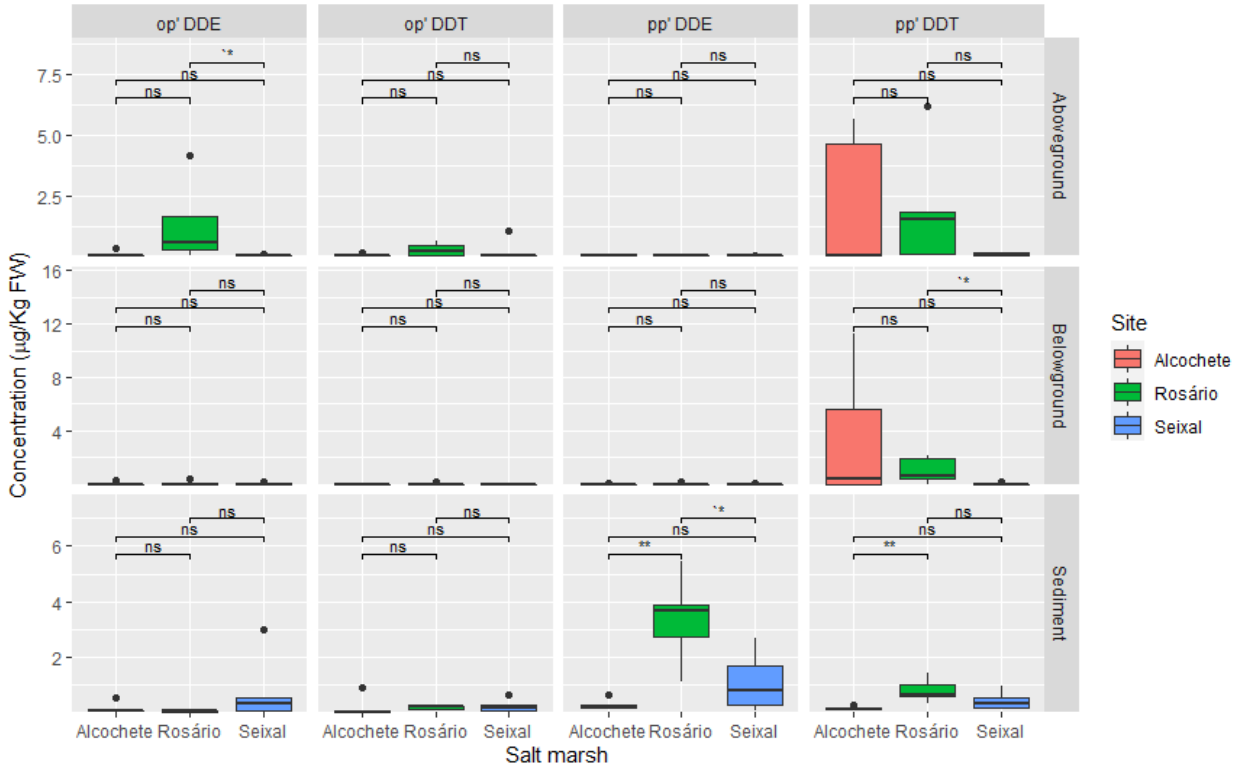


*Figure 2.5- Boxplot's representative of the results of the pesticides analysed in this work, presented in the plant (aboveground and belowground) and in sediments' samples sampled in Alcochete, Rosário, and Seixal. With the respective medium, acceptance intervals (25-75% range) and outliers. N=5. NS means non-significance and the asterisk represent statistically significant, with * represents p<0.05 and ** represents p<0.01.*

Figure 2.5 presents the interval of results in form of boxplots obtained from the quantification of pesticides extracted from sediments and plants aboveground and belowground levels.

In Figure 2.5 it is possible to verify a statistical difference ($p<0.05$) in the results of op'DDE between the Rosário and Seixal sites when studying the aboveground part of the plants, with the plants from the first site presenting higher op'DDE concentrations.

The belowground part of the plant presented a statistical difference ($p<0.05$) concerning the pp'DDT compound, between Rosário and Seixal sites, with the plants from the first site presenting higher pp'DDT relevance.

In the results obtained from the pp'DDE pesticide contained in the estuarine sediments, it was possible to notice a statistical difference between Rosário and Seixal ($p<0.05$) and Rosário and Alcochete ($p<0.01$). Rosário presents the highest concentration values of this compound. Regarding pp'DDT, the concentration of this compound in the sediments from Rosário was found to be significantly higher ($p<0.01$) than those detected in the sediments collected at Alcochete salt marsh.

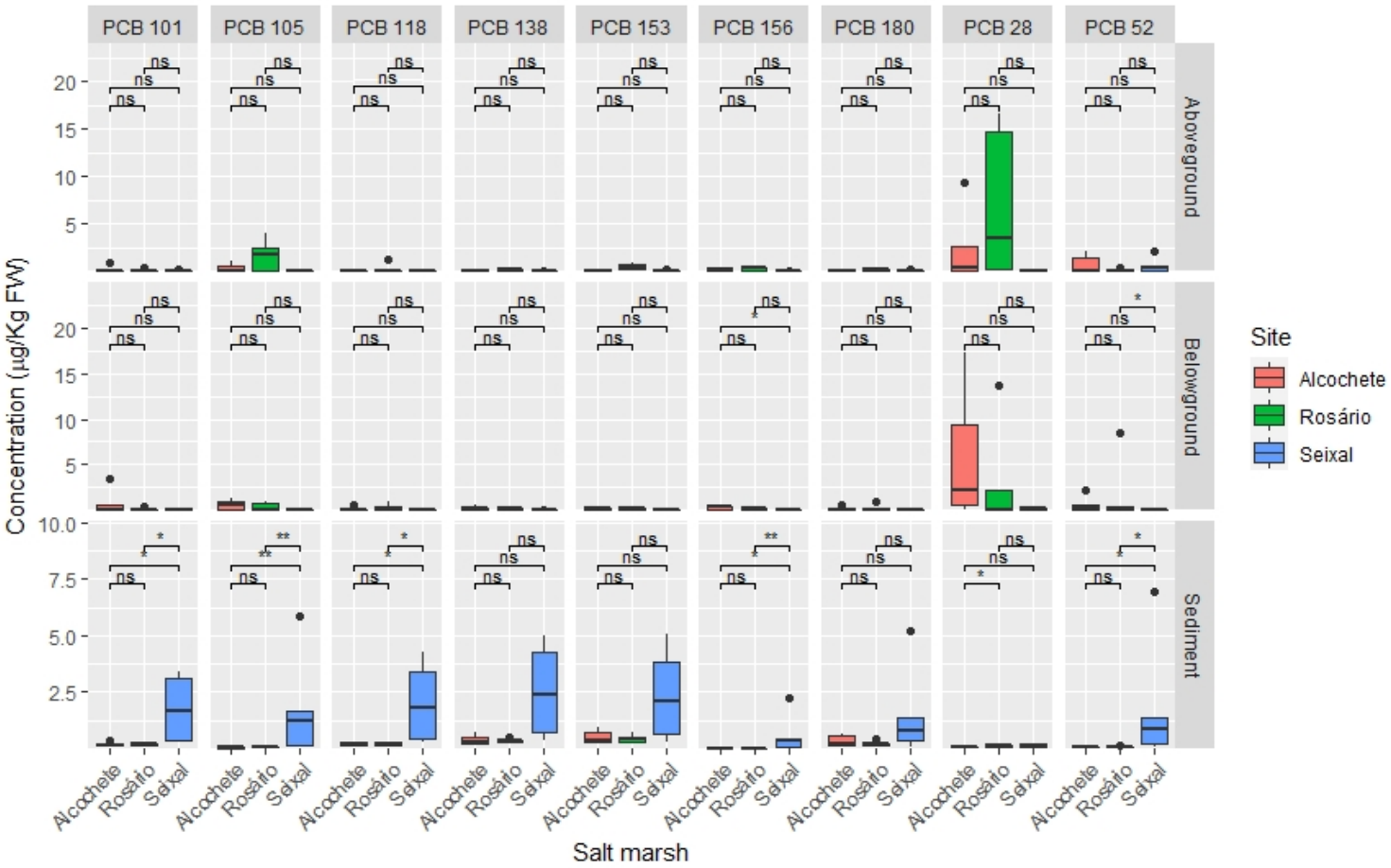


*Figure 2.6- Boxplot's representative of the results of the PCBs analysed in this work, presented in the plant (aboveground and belowground) and in sediments' samples sampled in Alcochete, Rosário, and Seixal. With the respective medium, acceptance intervals (25-75% range) and outliers. N=5. NS means non-significance and the asterisk represents statistically significant, with * representing p<0.05 and ** representing p<0.01.*

Figure 2.6 presents the concentration of the PCBs from the plants, below and above ground level, and sediments samples from the Alcochete, Rosário and Seixal's salt marshes.

In this figure, it is possible to verify a significant increase ($p<0.05$) when comparing the PCB 52's concentration values present in the belowground part of the plant, originated from the Rosário's salt marsh, with the same compound on the aboveground part of the plant sampled in Seixal. Also, in the belowground part of the plant, it was possible to observe a significantly higher concentration value of PCB156 from Alcochete's samples, when compared to the same compound presented in the belowground part of the plant originated from the Seixal's site.

Concerning PCB 101's concentration on sediments, it was possible to notice a significantly lower ($p<0.05$) concentration value of Alcochete and Rosário, when compared to the concentration of the same compound on Seixal's salt marsh. When observing the concentration of PCB 105's concentration on sediments from the Alcochete and Rosário's salt marsh, it is possible to notice that its concentrations values were significantly lower ($p<0.01$) than the ones obtained from the sediments derived from the Seixal site. This latter also presented significantly higher ($p<0.05$) values of concentration of PCB 118 and 52 when compared to the other two sites. Regarding the PCB 156's concentration presented in the sediments of the Seixal, it was significantly higher than those obtained for Rosário's sediments ($p<0.05$) and Alcochete ($p<0.01$).

Concerning the PCB 28's concentration values presented in the sediments that originated from Alcochete's salt marsh, they had significantly lower ($p<0.05$) concentration values than the ones presented in the sediments originated from Rosário's site.

2.5. Discussion

Table 2.5 presents the values of SQGs mentioned previously and the values of concentrations of the compounds analysed in the current work.

Table 2.5- Values of the SQGs mentioned by US EPA [43], OSPAR [44] and Wisconsin Department [45] and the interval of results obtained in this thesis for each site 's sediments. ERL means effects range low, BC/LC means background/low concentrations, BAC represents background assessment concentration, EAC represents the environmental assessment criteria, TEC means the threshold effect concentration and PEC is the probable effect concentration.

	US EPA (µg/kg)	**OSPAR (µg/kg)**			**Wisconsin Department (µg/kg)**		**Alcochete**	**Rosário**	**Seixal**
	ERL	BC/LC	BAC	EAC	TEC	PEC	Interval of concentrations (µg/kg)	Interval of concentrations (µg/kg)	Interval of concentrations (µg/kg)
PCB 28	-	0.0/0.05	0.22	1.7	-	-	≤0.070; 0.094 ± 0,024	0.081 ± 0.020; 0.151 ± 0.038	≤0.070; 0.25 ± 0.63
PCB 52	-	0.0/0.06	0.12	2.7	-	-	≤0.070; 0.101 ± 0028	≤0.070; 0.109 ± 0.030	0.096 ± 0.026; 6.9329 ± 1.9
PCB 101	-	0.0/0.07	0.14	3	-	-	0.115 ± 0,030; 0.341 ± 0,090	0.107 ± 0.028- 0.311 ± 0.082	0.302 ± 0.080; 17.3 ± 4.6
PCB 118	-	0.0/0.08	0.17	0.6	-	-	0.118 ± 0.031; 0.277 ± 0.072	0.159 ± 0.041; 0.325 ± 0.085	0.308 ± 0.080; 16.1 ± 4.2
PCB 138	-	-	-	-	-	-	0.225 ± 0.063; 0,76 ± 0,21	0.273 ± 0.077; 0.53 ± 0.15	0.37 ± 0.11; 19.5 ± 5.5
PCB 153	-	0.0/0.10	0.19	40	-	-	0.208 ± 0.056; 0.92 ± 0.25	0.309 ± 0.083; 0.72 ± 0.19	0.318 ± 0.086; 17.4 ± 4.7
PCB 180	-	0.0/0.11	0.1	12	-	-	0.084 ± 0.023; 0.67 ± 0.18	0.133 ± 0.036; 0.41 ± 0.11	0.077 ± 0.021; 5.2 ± 1.4
Σ PCBs	11.5	0.2	0.46	-	60	676	0.85 ± 0.11; 3.15 ± 0.39	1.09 ± 0.13; 2.52 ± 0.30	1.48 ± 0.18; 82.6 ± 9.8
pp' DDE	2.2	0.0/0.05	-	-	-	-	0.129 ± 0.045; 0.64 ± 0.23	1.13 ± 0.40; 5.4 ± 1.9	<0,050 ; 2.71 ± 0.95
pp' DDT	-	-	-	-	-	-	≤0.050; 0.276 ± 0.097	0.33 ± 0.12; 1.41 ± 0.49	0.097 ± 0.034; 0.96 ± 0.34
Σ DDTs	1.58	-	-	-	4.2	63	0.0662-1.1854	0.4232-1.6699	0.1150-1.2293
Σ DDEs	-	-	-	-	3.2	31	0.3900-2.1000	1.1686-5.5107	0.1258-5.6843

By analysing the SQGs and the values obtained in the present work, it was possible to observe that Alcochete salt marsh sediments contained higher concentrations than the ones recommended by the OSPAR, in the following compounds: PCB 101, 118, 153, 180 and the total of PCBs.

The concentrations' values obtained for Rosário salt marsh sediments surpassed the recommended in the same compounds. Furthermore, these sediments also contained concentrations of pp' DDE and DDT higher than the SQGs proposed by the US EPA and

Wisconsin Department. It was also possible to observe that the DDE concentration presented in the sediments surpassed the TEC values obtained in DDE compounds.

The Seixal salt marsh sediments presented higher contaminations of PCB 28, 52, 101, 118, 153, 180 and the total of PCB compounds than the values suggested by OSPAR and Wisconsin parameters. It was also possible to notice that the sediment concentrations values for pp' DDE surpassed the SQGs values of US EPA's and, in the case of the concentration of the DDE compounds presented in the sediments, it was higher than the ones purposed by OSPAR.

It was possible to observe that the Rosário salt marsh sediments had higher concentration values of pesticides, and Seixal salt marsh sediments had a predominant concentration of PCBs compounds. It was also possible to state that Alcochete had a smaller number of compounds that surpassed the SQGs.

Regarding the sediments from Alcochete, it was possible to observe that there was a higher percentage of grains with a bigger size than the sediments, represented in their percentage of gravel, that originated from the rest of the two marshes. This fact translates into a lower concentration of organic compounds, as could be seen in the values of TOC and LOI, which have the minimum values of both parameters [57]. The grain size and organic compound concentration difference could be derivative of its maturity. Since Alcochete marsh is recent, it is not very mature, containing grains of sediments with bigger size and, consequently, less organic compounds [58]. Another reason could be that this marsh is more upstream than the other two sites, which leads to a greater deposition of heavier sediments, this is, sediments with bigger grain, because the river loses its energy gradually from upstream to downstream [59].

In table 2.6, we present the values obtained in this thesis and the values obtained in other works when studying organochloride pesticides concentration and PCBs in sediments with origin from Portuguese estuaries: Carvalho, (2009) [60], which studied the concentrations of DDE and DDT compounds presented in the sediments present in the following Portuguese estuaries: Minho, Lima, Cávado, Ave, Douro, Sado and Ria Formosa [60]; Vale, (1999) the concentration of pp' DDE and pp' DDT compounds presented in the sediments sampled in the lower part of the Estuary of the Tagus and the upper Sado Estuary [61]; Mil-Homens, (2016) which studied the concentrations of PCBs in the Tagus prodelta's sediments observed results of a total of seven PCBs (the same ones analyzed in the present work); and Lobo, J., (2010), that studied PCBs concentration and pp' DDT and pp' DDD, on Sado's estuarine sediments,

Tabel 2.6- Concentration's interval (in µg/kg) of PCBs and pp' DDE and pp' DDT compounds obtained in this thesis for each site's sediments and for other works that studied PCBs and organochloride pesticides in Portuguese estuaries sediments, obtained in Carvalho, P. et al., (2009), Vale, O. (1999), Mil-Homens, (2016) and Lobo, J., (2010) [60-63].

	Alcochete	**Rosário**	**Seixal**	**Carvalho, P. et al., (2009) [60]**	**Vale, O. (1999) [61]**		**Mil-Homens, (2016) [62]**	**Lobo, J. et al., (2010) [63]**
	Interval of concentrations (µg/kg)	Interval of concentrations (µg/kg)	Interval of concentrations (µg/kg)	Douro, Minho, Lima, Ave, Cávado and Ria Formosa (µg/kg)	Tagus (µg/kg)	Sado (µg/kg)	Tagus (µg/kg)	Sado (µg/kg)
PCB 28	≤0.070; 0.094 ± 0.024	0.081 ± 0.020; 0.151 ± 0.038	≤0.070; 0.25 ± 0.63	-	-	-	-	-
PCB 52	≤0.070; 0.101 ± 0.028	≤0.070; 0.109 ± 0.030	0.096 ± 0.026; 6.9329 ± 1.9	-	-	-	-	0.08; 0.5
PCB 101	0.115 ± 0.030; 0.341 ± 0.090	0.107 ± 0.028; 0.311 ± 0.082	0.302 ± 0.080; 17.3 ± 4.6	-	-	-	-	0.06;1.2
PCB 118	0.118 ± 0.031; 0.277 ± 0.072	0.159 ± 0.041; 0.325 ± 0.085	0.308 ± 0.080; 16.1 ± 4.2	-	-	-	-	0.1;2.7
PCB 138	0.225 ± 0.063; 0.76 ± 0.21	0.273 ± 0.077; 0.53 ± 0.15	0.37 ± 0.11; 19.5 ± 5.5	-	-	-	-	0.1
PCB 153	0.208 ± 0.056; 0.92 ± 0.25	0.309 ± 0.083; 0.72 ± 0.19	0.318 ± 0.086; 17.4 ± 4.7	-	-	-	-	0.1;3.4
PCB 180	0.084 ± 0.023; 0.67 ± 0.18	0.133 ± 0.036; 0.41 ± 0.11	0.077 ± 0.021; 5.2 ± 1.4	-	-	-	-	0.1;0.6
Σ PCBs	0,85 ± 0,11; 3,15 ± 0,39	1,09 ± 0,13; 2,52 ± 0,30	1,48 ± 0,18; 82,6 ± 9,8	-	-	-	0.3-30.6	-
pp' DDE	0.129 ± 0.045; 0.64 ± 0.23	1.13 ± 0.40; 5.4 ± 1.9	<0.050; 2.71 ± 0.95	-	0.02;10.5	0.02; 2.13	-	-
pp' DDT	≤0.050; 0.276 ± 0.097	0.33 ± 0.12; 1.41 ± 0.49	0.097 ± 0.034; 0.96 ± 0.34	-	0.07;10.1	0.07;0.99	-	-
Σ DDTs	0.0662;1.1854	0.4232;1.6699	0.1150;1.2293	0.27;3.9	-	-	-	-
Σ DDEs	0.3900;2.1000	1.1686;5.5107	0.1258;5.6843	0.29;2.6	-	-	-	-

The values obtained for DDE in the present work were higher than the ones obtained by Carvalho, P. (2009), especially in the sediments collected at Rosário and Seixal. The samples collected at Alcochete are within the ranges previously reported. All the values obtained for DDT compounds were present in the respective interval or lower. Also, in the previously mentioned work, it was possible to correlate a higher concentration of these pesticides with surrounding agricultural activities, which corroborates the results of the current work, with Rosário displaying several agricultural fields in its vicinity and a higher pesticide concentration.

When comparing the results obtained in this thesis, it is possible to notice that the results were within the range obtained by Vale, O., (1999) [61]. In the case of Sado Estuary sediments, the

results obtained in the work mentioned varied between 0.02 to 2.13 μg/kg for pp' DDE compound and 0.07 to 0.99 μg/kg for pp' DDT compound. In this case, it was possible to observe that Rosário and Seixal's concentration of the compounds pp' DDE and pp' DDT were higher than those obtained in Sado [61].

With these comparisons between the results obtained in this work and the ones obtained in other works, it is possible to state that the Tagus salt marsh sediments were more polluted, since they have higher concentration of pesticide compounds than the other estuaries. This is due to the fact that the Tagus estuary has a higher percentage of the population and consecutively a higher activity of agriculture, empowering toxic effects in its ecology.

Mil-Homens, (2016)'s works had similar trend to the one observed in the present thesis with PCBs 153 and 138 being the ones that presented higher concentrations [62].

When comparing the results to Lobo, J., (2010), it is possible to notice that Alcochete and Rosário had lower values, presented in their sediments, than the ones obtained in this work, except for PCB 180 in Alcochete. Concerning Seixal's sediments, all the analysed PCBs had higher values than the ones obtained in this thesis, so it is possible to determine that Seixal is more polluted. Regarding pp' DDE, Rosário and Seixal's sediments had a higher concentration of these compounds. The results obtained for pp' DDT pesticide of this mentioned work were higher than the ones obtained in this thesis [63].

The PCB concentration result was higher in Seixal when compared to other sites. This agrees with the industrial history of the Seixal area, since PCBs were commonly used in industries such as paper, electronics, and others [3, 11, 12], being Seixal the most industrialized site surveyed in the present work.

In this work, it was possible to notice several outliers in the boxplot graphs. These outliers are derivative of the environmental variability and not analytical variability. This could be explained by the fact that sediments are a type of matrix that contains different sizes of grains, which can influence these results.

All these results corresponded to the state of the site and the type of activity occurring there. Alcochete is a more upstream site, and there are few agricultural and industrial activities. Rosário is a site where a lot of agricultural activity exists, and this activity was known to use pesticides such as DDTs to control the pests and insects that affect the crops. Since Seixal is an industrial site, PCBs were used to help the work happening in the paper, electronic and other industries.

Few scientific works study the concentrations of these compounds in plants. One of these works is the one made by Cardoso (2013). In this work, the concentrations of PCBs (in this case dioxin-like PCBs) were studied in two species of halophyte vegetation: *Sarcocornia perennis* and *Halimione portucaloides*. It was concluded that even though the contaminants are transferred from the rhizosediments to the aboveground tissues, this occurs in reduced amounts. The belowground plant has a higher concentration of contaminants than the aboveground [19]. In this work, however, we were unable to reach a conclusion, since there was in some cases parts of plants sampled in the same site with high concentrations of the same compound, such as the case of pp' DDT compound originated from Alcochete, so it is possible that there was a transport of this compound from belowground to aboveground.

2.6. Conclusions

In the current work, it was possible to observe that, even though the organochloride compounds were banned in the 1990s (in the case of DDT it was banned in the 1970s), compounds like these were still found in Alcochete, Rosário and Seixal salt marsh.

It was also possible to conclude that Rosário was the marsh with the highest concentration of pesticides and Seixal had higher concentrations of PCB compounds present in its sediments. This is representative of the activity that occurs in the zone. Rosário presents a predominant agriculture activity, that in the past used these compounds. Seixal is an industrial zone that used PCBs compounds. The sediments originated from Alcochete presented the lowest values of concentration of these compounds, when compared to the values obtained on the other sediments marshes analysed in this work. This has a direct connection to the sediments' characteristics, since it presented a bigger grain size of sediments and low LOI and TOC.

When comparing the results obtained from the analysis of the sediments of this work with similar works done in different sites, it could be observed that the marshes analysed in the current work were polluted, mainly because of their greater concentration of population.

When observing the results of the pesticides and PCBs' concentration on the *Spartina maritima* plant, it was possible to notice a possible transportation of the compounds analysed from belowground to aboveground.

3. Ecotoxicity of PCB 153 in the model marine diatom *Phaeodactylum tricornutum*

3.1. Introduction

Emerging pollutants are compounds that have anthropogenic or natural origins which cause or are suspected to cause toxic effects on ecology or human health. Some examples of such compounds are the ones mentioned in this thesis, the organochloride pesticides and PCBs compounds [64].

The PCB 153, or 2,2',4,4',5,5'-hexachlorobiphenyl is one of the compounds that is included in this category and that can be found in environmental samples, as described in the previous chapter. This non-dioxin-like, di-ortho-substituted PCB is the most predominant in marine biota, having also been detected in high concentrations in the environment and human serum [65]. According to Monikh, (2013), it was proven that this pollutant can be transferred in the food chain and biomagnified along with the trophic segments [66], leading to health issues in humans, associated with inflammation: cancer, metabolic syndrome, and endocrine dysfunction [67, 68].

The mechanism of its toxicity is documented in Livingstone (2001), in which the PCB compound causes the cytochrome P450 system to suffer uncoupling, producing O_2^- and H_2O_2. Since these are reactive oxygen species (ROS), it will induce oxidation in the organism [69].

To evaluate the impacts of the environmentally detected PCB concentrations in the marine autotrophic biota, an ecotoxicity assay was performed in the present work using a marine model diatom as the target species to disclose the effects of this compound in this key organism for the estuarine system. These organisms were chosen in a range of size between 0,2-200 μm and high uptake rates, being at the base of the food chain. As such, any difference that occurs in this species will affect the system that they are present [70]. More specifically, in the present work, a model diatom was used, *Phaeodactylum tricornutum*. This diatom is currently used in stress and ecotoxicological studies (such as the effects of temperature [71, 72], heavy metal exposure [73, 74], nutrient depletion [75] or emerging pollutants [76, 77]), due to their environmental spread and their early reaction that reflects a sign of biological stress [78, 79].

Several bibliographic sources focused on the exposure of marine biota to pesticides and other organochloride compounds. In these tests, the authors recur to acute toxicity tests, i.e., short-term tests where a target model species is exposed to a certain contaminant within environmentally relevant concentrations. To study the effects of the contaminants in the target organism, certain

biomarkers (enzymatic and non-enzymatic) are also usually analysed. In the case of photosynthetic beings, like the diatom studied in this project, its photochemistry and the sinthesized pigments are also analysed [70, 80, 81]. The photochemistry analysis focused on certain biomarkers that study the efficiency of the photosynthesis of this diatom.

Photosynthesis is a procedure that occurs in the chloroplast, more specifically in the chloroplast stroma of plants and photosynthetic organisms. In this section of the chloroplast, light is absorbed by the photosystem I (PSI) and photosystem II (PSII), reaching its reaction centres where the principal reactions of this procedure occurs. The photosynthesis activity starts with the PSII transforming water into molecular oxygen and two hydrogen cations. These cations will reduce a biomolecule called plastoquinone (PQ) into plastoquinol (PQH_2) and carry the protons into the PSI, degrading into PQ. PSI will receive the proton, and with the light received, it will transform NADP into NADPH, which is necessary for several different processes within the plant [82].

Superoxide dismutase, catalase, or ascorbate peroxidase (or, SOD, CAT and APx, respectively) are enzymes used as a natural mechanism on living beings to defend against reactive oxygen species (ROS), which is induced by the interaction between the organisms and contaminants. Examples of ROS are superoxide, hydrogen peroxide and others [31]. These components, when accumulated within an intracellular environment can induce biochemical damage by oxidizing certain biomolecules such as lipids, proteins, and others, and even altering the metabolization of the cell [83].

The objective of this work was to study the ecotoxicity of the PCB 153 contaminant on the diatom species, *Phaedactylum tricornutum*, by evaluating its impact on growth, photosynthesis efficiency, pigment concentration and antioxidant enzymatic activities.

3.2. Materials and methods

3.2.1. Experimental setup

Ecotoxicological exposure tests were performed using *P. tricornutum* Bohlin (Bacillariophyceae) (IO 108–01, IPMA, ALISU—Algae Collection of the University of Lisbon, Lisbon, Portugal). Cultures were maintained in 250 mL of f/2 medium in culture flasks under controlled conditions for 4 days (preserved in a growth chamber, Fytoscope FS130 at 18 ± 1 °C, with constant aeration and 14 hours of light/10h dark photoperiod, with a light intensity of 80 μmol photons m^{-2} s^{-1}). The growth chamber was programmed to simulate a natural light environment, with light intensity to simulate natural sunrise and sunset [65, 71, 84-86].

According to the guidelines for algae bioassays presented by the Organization for Economic Cooperation and Development (OECD), the initial cell concentration presented in the cultures was $2.7x10^5$ cells mL^{-1} (OECD, 2002) [87]. After 48 h, the samples were inoculated with PCB 153 at different concentrations: 0 μg/L (denominated control culture), 1 μg/L, 3 μg/L and 6 μg/L. Each one of these concentrations was applied in triplicates, making a total of 12 experimental units. These concentrations were chosen according to the range of results that were obtained in this work when analysing the concentration of pesticides and PCBs in an estuary environment.

The exposure occurred for 48 h. All culture manipulations were performed in a laminar airflow chamber, under standard aseptic conditions [64, 71, 84, 85].

3.2.2. Cell Growth rate and pellet collection

To analyse the cell density, a FluoroPen FP100 (Photo System Instruments, Czech Republic) was used, as well as a cuvette with 1 mL of sample, by applying a non-actinic light (Ft) in a dark-

adapted environment. This procedure was done daily to quantify the cell density of the different treatments [65, 84, 86]. To calculate the concentration of the cells in their culture, the following equation was used, resultant from a previous calibration performed for this device and this species:

$$C_{cell} = (3429 \times [Ft]) - 976200 \quad (3.1)$$

Being:

- C_{cell} – The concentration of the cell on its culture (cell/mL)
- [Ft] – The result obtained by the quantification of chlorophyll fluorescence

The specific growth rate (SGR) was calculated by subtraction between initial (Ci) and final (Cf) logarithmic concentration divided by the number of exposure days:

$$SGR = \frac{\ln(Ci) - \ln(Cf)}{number\ of\ days} \quad (3.2)$$

After 48 of exposure time with the PCB, samples were collected for biochemical and molecular analyses. The cells were collected from their culture's flask, centrifuged at 4000 *g* for 15 min at 4 ºC and after supernatant removal, the cells pellets were frozen at -80 ºC until analysis.

3.2.3. Chlorophyll a Pulse Amplitude Modulated Fluorometry

To evaluate diatom photochemical features, Pulse Amplitude Modulated (PAM) fluorometry was performed using a FluoroPen FP100 (Photo System Instruments, Czech Republic) on 15 min dark-adapted using 1 mL of sample on a cuvette. Chlorophyll transient light curves (Kautsky plot) were conducted using the OJIP test, according to Duarte, B., (2019). This test allows the dermination of multiple variables [65, 71, 84, 85], according to Table 3.1 [76].

Table 3.1- Variants quantified in the analysis of the OJIP test and its description.

Parameters	Description
Mo	Net Rate of PSII reaction centres closure
Area	Corresponds to the oxidized quinone pool size available for reduction
N	Reaction centre turnover rate
S_M	Corresponds to the energy needed to close all reaction centres
P_G	Grouping probability between the two PSII units
ABS/CS	Absorbed energy flux per cross-section
TR/CS	Trapped energy flux per cross-section
ET/CS	Electron transport energy flux per cross-section
DI/CS	Dissipated energy flux per cross-section
RC/CS	Number of available reaction centres per cross-section

δR_0	The efficiency of the transfer of an electron from PQH_2 to final PS I acceptors
SFI	Structure functional index for photosynthesis
SFI (NPQ)	Non-photochemical quenching or dissipation structure-function
RE_0/RC	Electron transport from PQH_2 to the reduction of PS I end electron acceptors
RC/ABS	Reaction centre II density within the antenna chlorophyll bed of PS II

3.2.4. Diatom uptake

The QuEChERS procedure described before was employed to extract the PCB 153 from the diatom cells. First, the cells' pellets were tranferred to a 50 mL tube, followed by the addition of 15 mL 1% acetic acid in acetonitrile. The extraction and the clean-up salts mixtures were the same used in the procedure mentioned previously (for extraction 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 1 g of monosodium citrate and 0,5 g sodium citrate dibasic sesquihydrate and clean-up it was used 2,5 mg of GCB and 25 mg of PSA). In both extraction and clean-up procedures, the samples were centrifuged in a vortex at 1500 rpm for 1 min procedure. Next, the sample was let until almost dry at a nitrogen flow. Finally, 1 mL of isooctane was added. To analyse the PCB content present intracellularly in the cells, we recurred to the GC-ECD technique, using the same conditions of the analysis of PCBs and pesticides on plants, in Table 2.3 [52].

3.2.5. Diatom pigment profiles

Pigment quantification was performed after extraction of the cell pellets with pure acetone to the sample pellets. They were maintained in a cold ultra-sound bath for two minutes to guarantee that the cell composition was completely disaggregated. They were extracted at -20 ºC for 24 h in the dark to prevent degradation. Then, it was centrifuged at 4000 g and 4 ºC for 15 min [71]. Finally, the supernatant was scanned in a dual-beam spectrophotometer from 350 nm to 750 nm at 0,5 nm steps. Then, these results were introduced in Gauss-Peas Spektra (GPS) library using SigmaPlot Software. The concentration of Chlorophyll a and c, Pheophytin a, β-carotene, Fucoxanthin (Fx), Diadinoxanthin (Ddx) and Diatoxanthin (Dtx) were quantified using the algorithm developed by Küpper *et al.* (2007) [88]. Finally, the De-Epoxidation State (DES) was calculated using the equation:

$$DES = \frac{Dtx}{Dtx+Ddx} \quad (3.3)$$

3.2.6. Lipid peroxidation products quantification

To determine the quantity of lipid peroxidation products, we recurred to the same procedure used by Heath and Packer (1968), in which the cell pellet was homogenised with 1.5 mL of a solution of col 10 (v/v) trichloroacetic acid (TCA) and put in a cold ultra-sound bath for 1 min and, afterwards, at 100 ºC for 30 min. Following these steps, the samples were centrifuged at 15000 g for 10 min at 4 ºC. Then 1 mL of the supernatant was added to 1 mL of thiobarbituric acid (TBA) and heated for 30 min at 95 ºC. Then, it was cooled down and centrifuged just as done previously in this procedure. Finally, the absorbance was read at 532 and 600 nm by spectrophotometry [89]. The concentration of malondialdehyde (MDA) was calculated using the molar extinction coefficient, 155 mM^{-1} cm^{-1}.

3.2.7. Antioxidant enzyme activity measure

The protein fraction was extracted by adding to the sample pellets 1 mL of 50 mM sodium phosphate buffer (pH 7.6) with 0.1 mM Na-EDTA and followed by sonification for 1 min and centrifugation at 9000 rpm for 10 min at 4 ºC. Protein concentration was determined according to Bradford (1976) [90].

Catalase (CAT) activity was analysed by adding H_2O_2 and its consequent decrease at 240 nm (ε= 39,4 $mM^{-1}cm^{-1}$) for two minutes, in a medium composed of 188.8 µL of 50 mM of sodium phosphate buffer (pH 7.6), 10 µL of cell extract and 1.2 µL of 100 mM of H_2O_2 (15% v/v) [91].

Ascorbate peroxidase (APX) was determined by adding 10 µL of cell extract, 187 µL of 50 mM of sodium phosphate buffer (pH 7.0), 1 µL of 50 mM ascorbate and 10 mM of H_2O_2. The reaction was monitored at 290 nm (2.8 $mM^{-1}cm^{-1}$) through the decrease in the absorbance of the reaction mixture [92].

Superoxide dismutase (SOD) was assessed by adding 10 µL of cell extract, plus 110 µL 50 mM of sodium phosphate buffer, 72 µL of Milli-Q water and 8 µL of 6 mM of pyrogallol. The reaction was monitored throughout a reduction in the absorbance at 325 nm, due to a reduction of the pyrogallol molecule concentration on the medium [93]. To calculate the activity of this enzyme, we evaluated substrate auto-oxidation, which were assayed without substrate.

The assays done in this work were performed at 25 ºC.

3.2.8. Statistical Analysis

Differences between treatments were evaluated using the Kruskal-Wallis test with Bonferroni correction. Spearman correlation tests were employed to evaluate possible correlations between the assessed variables. All statistical analysis was performed in R-Studio Version 4.1.2 using the agricolae and corrplot packages, respectively for the Kruskal-Wallis test and Spearman correlation test.

3.3. Results

3.3.1. Cell Growth Rates and cell uptake

In Figure 3.1, it is possible to observe the concentration of the contaminant that was presented in the cells, i.e., the concentration that was uptake. It could be noticed that there was a significant difference between all the cultures exposed to different concentrations of PCB 153, with an increase in the exogenous PCB 153 concentration leading to significant increases in the intracellular concentration of this compound.

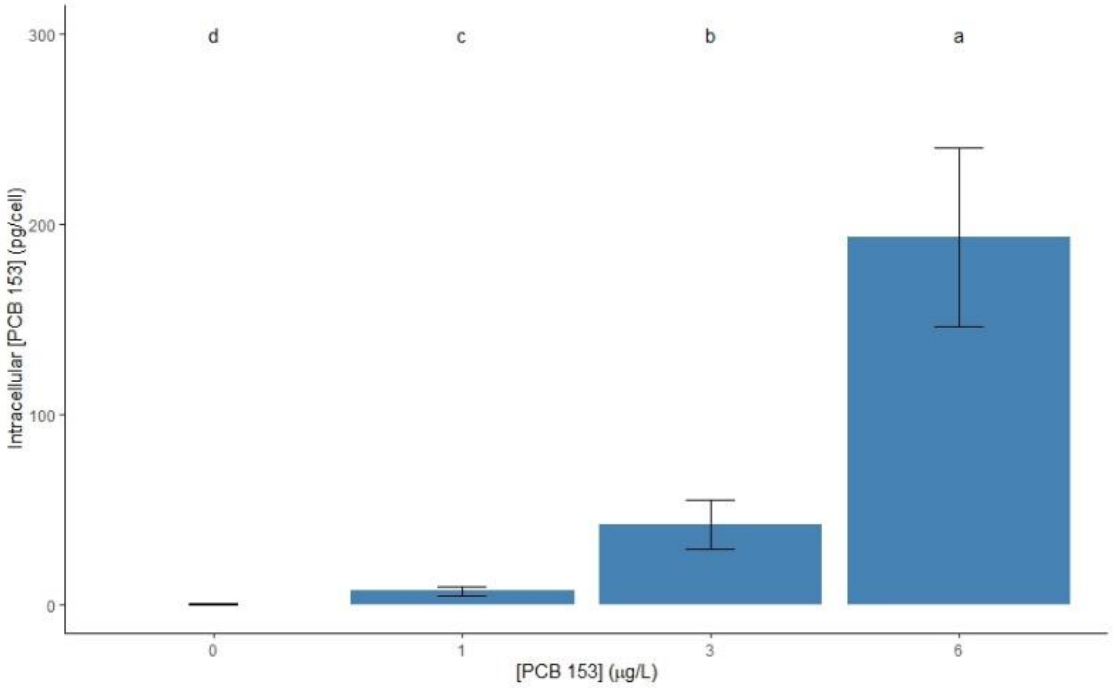


Figure 3.1- *Intracellular concentration of PCB 153 (pg/cell), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatment at p<0.05).*

*Figure 3.2- Specific Growth Rate (A), Growth Inhibition (B), Doubling time (C) and divisions per day (D), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).*Figure 3.1- *Intracellular concentration of PCB 153 (pg/cell), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatment at p<0.05).*

Regarding diatom-specific growth rate (Figure 3.2 A) and divisions per day (Figure 3.2 D), it could be observed that the two highest concentrations of PCB 153 tested led to significantly lower specific growth and divisions per day when compared to the values measured in the control and 1 µg/L PCB 153 subjected cultures.

By observing the diatom growth inhibition (3.2 B) and doubling time (Figure 3.2 C) it was possible to notice that there was a significant increase of these parameters in the cells exposed to the higher concentrations of the tested compound. In this case, it was possible to notice a higher value of growth inhibition of the two highest concentrations when compared to the control culture and the 1 µg/L PCB 153.

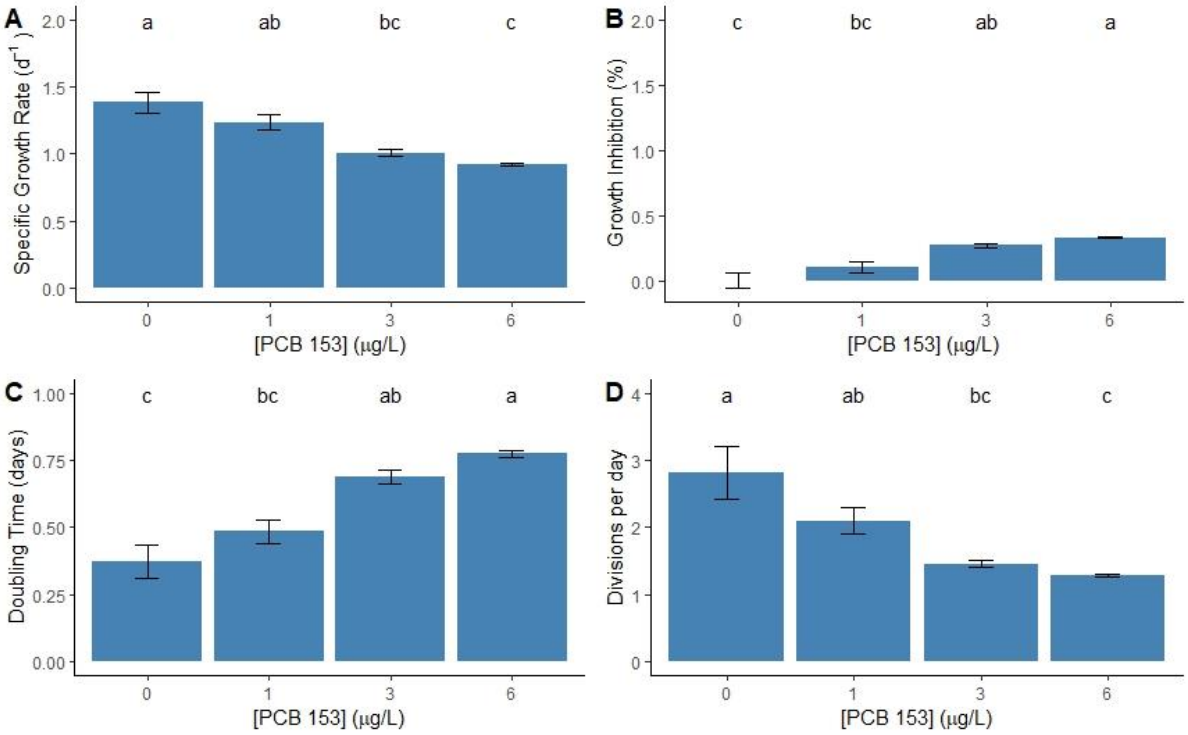


Figure 3.2- Specific Growth Rate (A), Growth Inhibition (B), Doubling time (C) and divisions per day (D), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).

In Figure 3.3 it is possible to notice a positive correlation between the concentration of PCB 153 presented in the cultures and presented intracellularly ($p<0.01$). When analysing this correlation, is possible to observe that specific growth rate (SGR) presented an inverse correlation with exogenous and intracellular PCB. The divisions per day (M) variant also showed a significant inverse correlation with both exogenous and intracellular PCB concentrations. The doubling time

(d) variant had a direct significant correlation with PCB concentrations, exogenous and intracellularly.

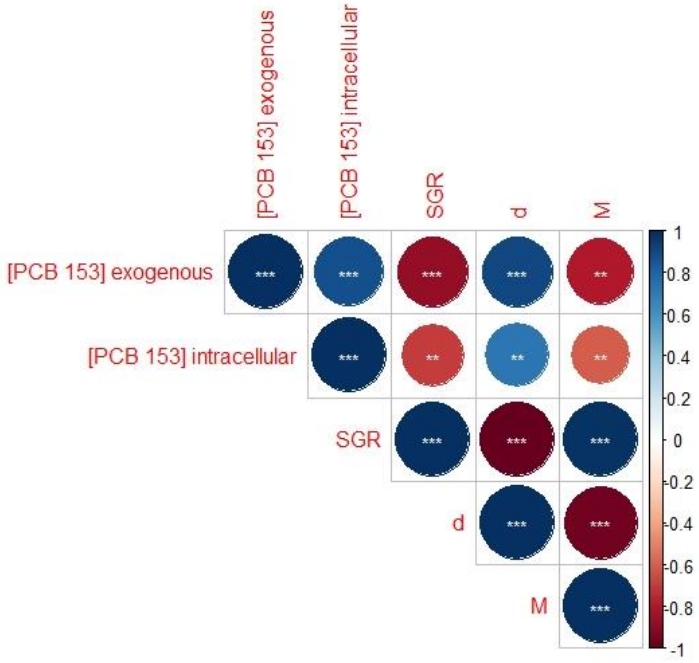


Figure 3.3- Correlation between the parameters Specific Growth Rate (SGR), Doubling time (d) and divisions per day (M), with the concentrations of PCB 153 that are presented exogenous and intracellular, in *Phaeodactylum tricornutum* following a 48 h exposure to PCB 153 in different concentrations (average ± standard error, n=3). *The darker the colour, and the bigger the dot the stronger is the correlation. When there is a * on a dot, it implies a statistical significance of p<0.05, ** p<0.01 and p<0.001.*

Figure 3.4- Net rate of PSII RC closure (Mo) (A), size of the oxidized quinone pool (B), energy to close all reaction centers (SM) (C) and reaction center turnover rate (N) (D), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).Figure 3.3- Correlation between the parameters Specific Growth Rate (SGR), Doubling time (d) and divisions per day (M), with the concentrations of PCB 153 that are presented exogenous and intracellular, in *Phaeodactylum tricornutum* following a 48 h exposure to PCB 153 in different concentrations (average ± standard error, n=3). *The darker the colour, and the bigger the dot the stronger is the correlation. When there is a * on a dot, it implies a statistical significance of p<0.05, ** p<0.01 and p<0.001.*

3.3.2. Diatom Photochemistry

Analyzing the net rate of PSII RC closure (Mo) (Figure 3.4 A), size of the oxidized quinone pool (3.4 B), energy to close all reaction centres (S_M) (Figure 3.4 C) and reaction centre turnover rate (N) (Figure 3.4 D), it is possible to notice that, in all the biomarkers presented, there was a significant decrease from the control culture in relation to the culture exposed to 6 µg/L of PCB 153.

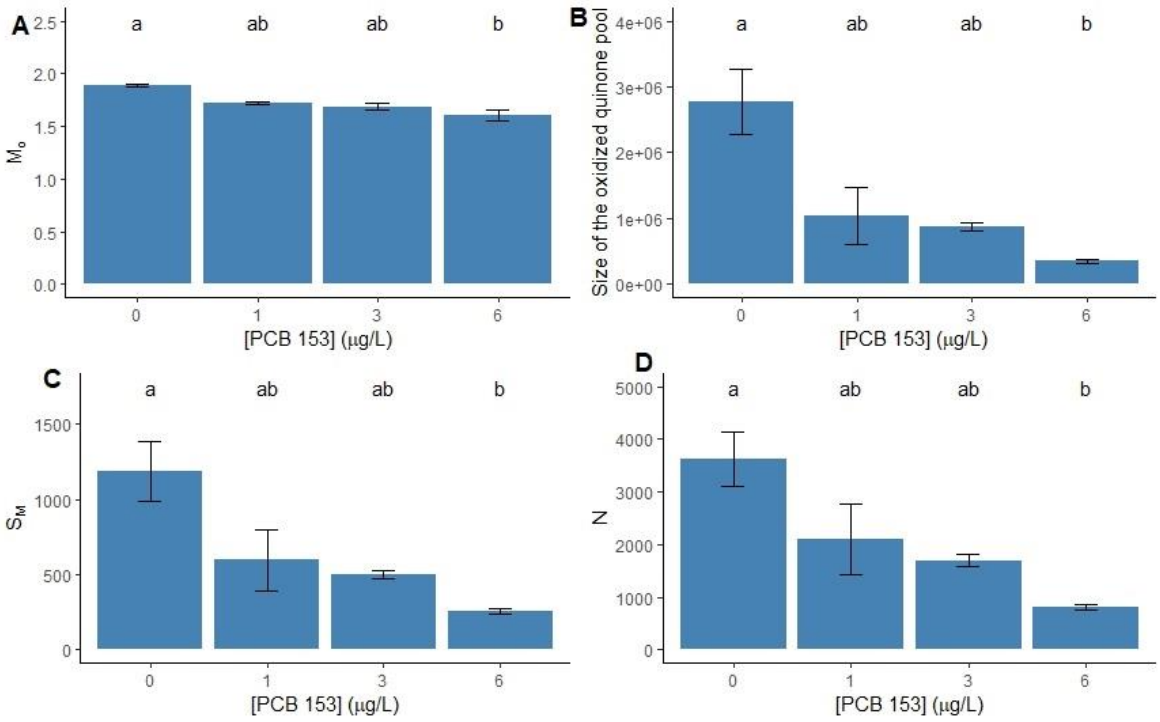


Figure 3.4- Net rate of PSII RC closure (Mo) (A), size of the oxidized quinone pool (B), energy to close all reaction centers (SM) (C) and reaction center turnover rate (N) (D), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).

Observing the ABS/CS (Figure 3.5 A), TR/CS (Figure 3.5 B), ET/CS (Figure 3.5 C) and RC/ABS (Figure 3.5 F), it was possible to notice there was a significant decrease in these photochemical variables from the control culture and 1 µg/L PCB 153 exposed to the culture and the cultures with the two highest concentration of PCB.

Regarding DI/IC (Figure 3.5 D), it is possible to notice a significantly lower value of this parameter when comparing the control culture with cells exposed to the highest concentration of PCB 153. Concerning the RC/CS (Figure 3.5 E), it was possible to observe a significant reduction of this variable with the increase of the PCB 153 concentration in the culture, and a significant difference between all the evaluated cultures exposed to different PCB 153 concentrations.

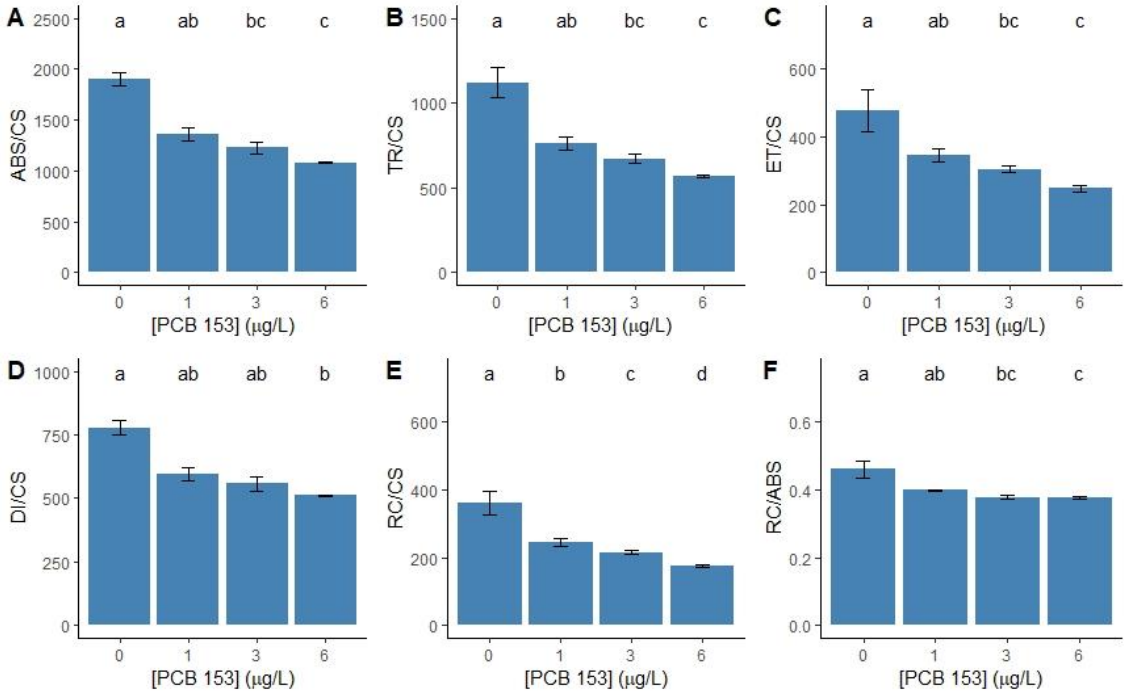


Figure 3.5- Absorbed energy flux per cross section (ABS/CS) (A), trapped energy flux per cross section (TR/CS) (B), electron transport energy flux per cross section (ET/CS) (C), dissipated energy flux per cross section (DI/CS) (D), number of available reaction centers (RC/CS) (E) and reaction center II density within the antenna chlorophyll bed of PS II (RC/ABS) (F), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different denote significant differences between treatments at p<0.05).

Regarding grouping probability (GP) (Figure 3.6 A), we verify that there was a significant increase in this parameter, especially in the cultures exposed to the highest PCB 153 concentrations. Comparing the 3 µg/L and 6 µg/L, a significant reduction in this value could be observed.

As for the the electron transport from PQH_2 to the reduction of PSI and electron acceptors (RE0/RC) (Figure 3.6 B) and efficiency of the transfer of the electron from PQH_2 to the final PSI acceptors (δRo) (Figure 3.6 C) parameters, it was possible to observe an increase of this parameter when comparing the control culture and the diatom cells exposed to 1 µg/L of PCB 153 with the cultures exposed to the highest concentration of PCB.

Concerning the non-photochemical quenching or dissipation structure functional index (SFI(NPQ)) value (Figure 3.6 E), it was possible to notice a significant increase in this parameter in the cultures exposed to 1 µg/L and 6 µg/L. The structure index for photosynthesis (SFI) (Figure 3.6 D) value showed the opposite trend, displaying significantly lower values in the cells exposed to the highest PCB 153 concentration tested.

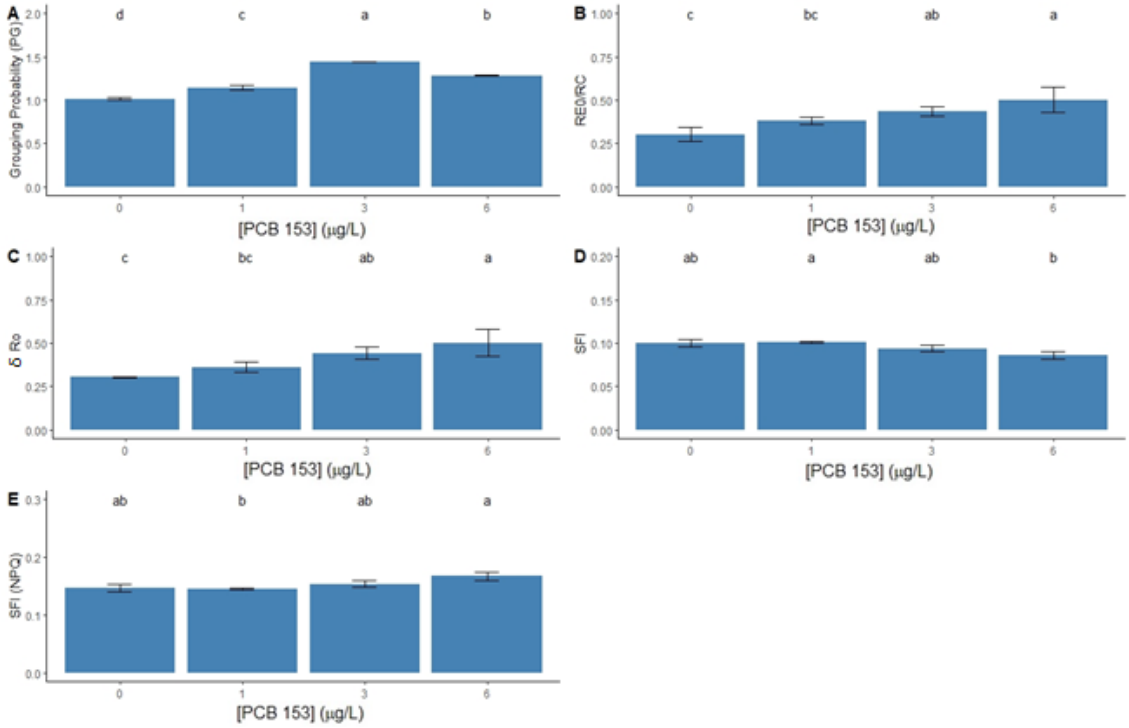


Figure 3.6- Grouping probability (A), electron transport from PQH2 to the reduction of PSI and electron acceptors (RE0/RC) (B), efficiency of the transfer of the electron from PQH2 to the final PSI acceptors (δRo) (C), structure functional index for photosynthesis (SFI) (D) and non-photochemical quenching or dissipation structure functional index (SFI(NPQ)) (E), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).

It was possible to notice an inverse significant correlation between exogenous and intracellular PCB 153 concentration and all photochemical parameters, with exception of Mo and intracellular PCB 153 concentration, which is shown in Figure 3.7.

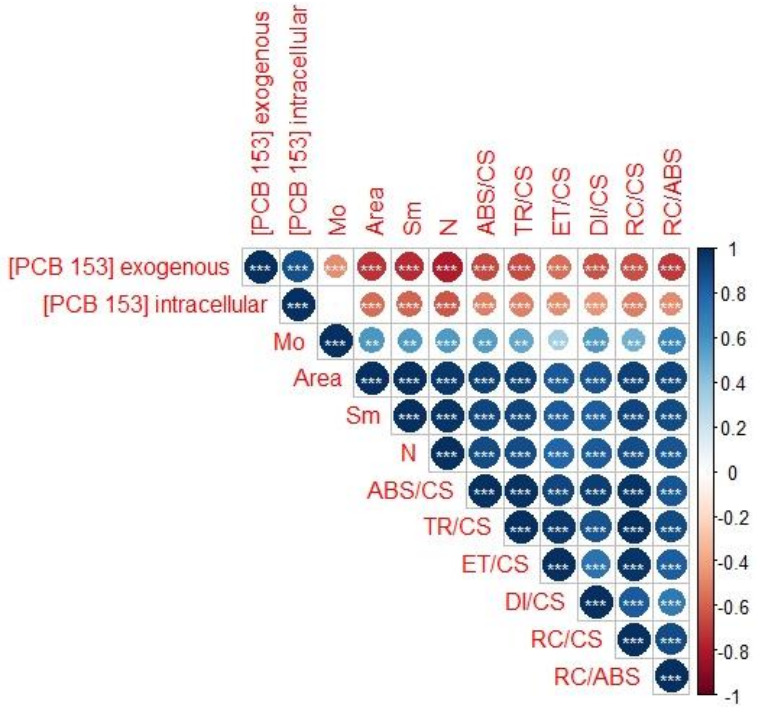


*Figure 3.7- Correlation between the parameters Mo, Area of oxidized quinone pool (Area), SM, N, ABS/CS, TR/CS, ET/CS, DI/CS, RC/CS, RC/ABS with the concentrations of PCB 153 that are presented exogenous and intracellular in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3). The darker the colour, and the bigger the dot the stronger is the correlation. When there is a * on a dot, It implies a statistical significant of $p<0.05$, ** $p<0.01$ and $p<0.001$.*

3.3.3. Diatoms' pigment profile

Regarding chlorophyll *a* (Chl *a*) and chlorophyll *c* (Chl *c*) (Figure 3.8 A and B, respectively), a significant decrease was noticeable between the cultures exposed to 1 µg/L of PCB 153 and the cultures that were exposed to the two highest concentrations of the tested compound.

Analysing the results of pheophytin a (Pheo *a*) and Fucoxanthin concentrations (Figure 3.8 C and E, respectively), it was possible to notice a significant increase between the cultures exposed with 6 µg/L of the tested compound and the control cultures/ cultures exposed to 1 µg/L of PCB 153.

When analysing diadinoxanthin (Figure 3.8 F) values of concentration, it is possible to notice that there was a significant increase between the control culture and the culture exposed to 3 µg/L of PCB 153. We also observe a significant difference between the culture exposed to 1 µg/L of PCB 153 and the cultures exposed with the highest concentration of the tested compound.

Regarding diatoxanthin (Figure 3.8 G), a significant increase was noticeable between the cultures exposed to 3 µg/L of PCB 153 and the cultures exposed to 6 µg/L of the exposed compound.

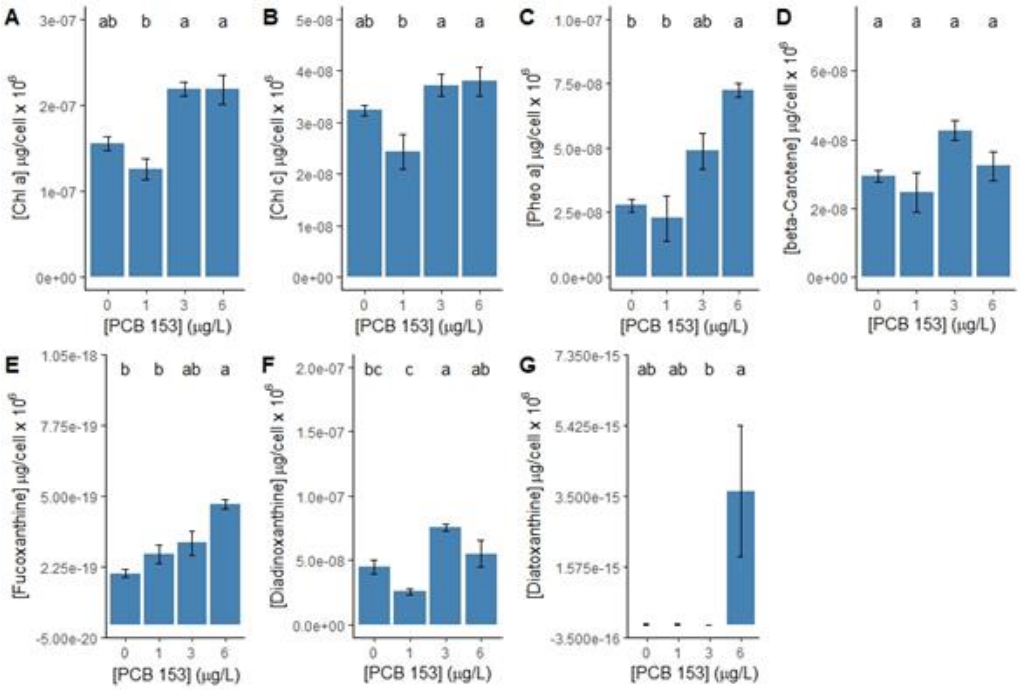


Figure 3.8- Chlorophyll a (Chl a) (A), Chlorophyll c (Chl c) (B), Pheo a (C), beta-carotene (D), Fucoxanthin (E), Diadinoxanthin (F), Diatoxanthin (G), in Phaeodactylum tricornutum following a 48 h exposure to differentconcentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).

The total carotenoid concentration (Figure 3.9 C) was the only parameter exhibiting significant differences between the tested treatments, with an increase in the concentration of these

compounds between the culture exposed to 1 µg/L and the cultures exposed to the highest concentration of PCB.

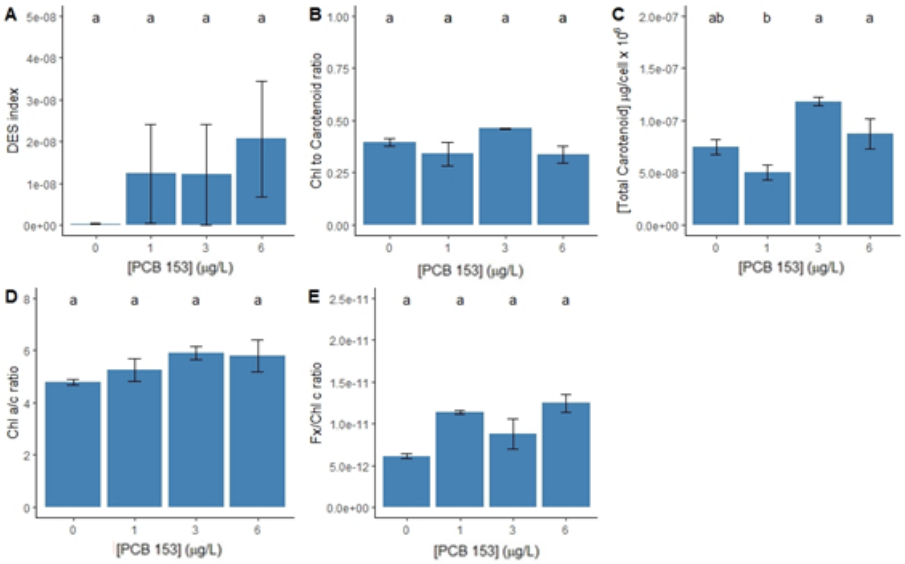


Figure 3.9- De-epoxidation state (DES) (A), Chlorophyll/Carotenoid ratio (B), Total Carotenoid (C), Chlorophyll a/c ratio (D) and Fucoxanthin/chlorophyll ratio (FX/Chl c ratio) (E), in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).

By analysing the correlations between the concentration of pigments present in the studied cells and the intracell and exogenous PCB concentration, it was possible to notice a direct correlation between the exogenous PCB and Chl *a* and *c*, which is shown in Figure 3.10.

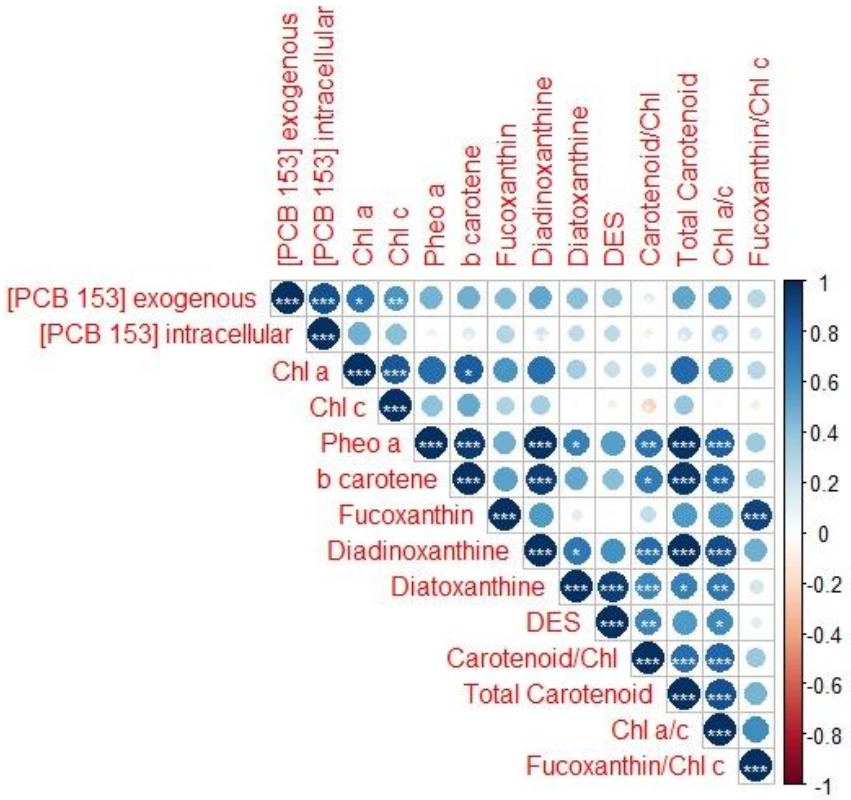


*Figure 3.10- Correlation between the parameters Chl a, Chl c, Pheo a, b-carotene, fucoxanthin, diadinoxanthin, diatoxanthin, DES, carotenoid/Chl ratio, total of carotenoid, Chl a/c and fucoxanthin/Chl c with the concentrations of PCB 153 that are presented exogenous and intracellular, in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard deviation, n=3, different letters signify a significant difference of $p<0.05$). The darker the dot the stronger is the correlation. When there is a * on a dot, It implies a statistical significance of $p<0.05$, ** $p<0.01$ and $p<0.001$.*

3.3.4. Antioxidant enzyme activity and lipid peroxidation

When analysing the results, it is possible to observe that catalase (CAT) (Figure 3.11 A) activity increased significantly when comparing the control culture and the culture with a higher concentration of PCB 153 compound, with the latter having a higher activity of this enzyme.

Ascorbate peroxidase (APx) activity (Figure 3.11 B) varied significantly between all the cultures, with the control culture displaying the lowest activity of this enzyme and the highest APx activity assessed in the cells exposed to 6 µg/L of PCB 153.

A significant increase in superoxide dismutase (SOD) (Figure 3.11 C) activity was also noticed in the culture control and the cultures exposed to 1 and 6 µg/L of PCB 153.

When observing the results of lipid peroxidation (Figure 3.11 D), no significant differences could be found between the different tested treatments.

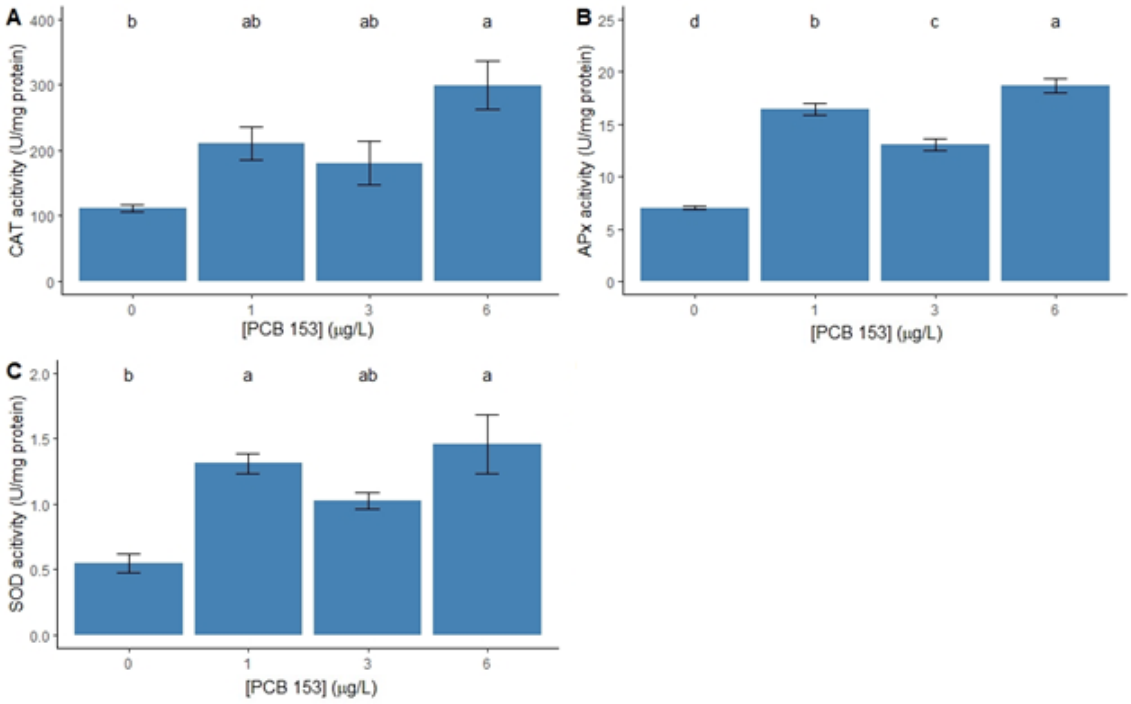


Figure 3.11- Catalase enzyme (CAT) (A), ascorbate peroxidase (APx) (B), superoxide oxidase (SOD), thiobarbituric acid (TBARS) in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters denote significant differences between treatments at p<0.05).

Observing the correlations between the oxidative stress biomarkers and the intracell and exogenous PCB 153 concentration, it was possible to notice that only APx activity revealed significant and direct correlations with the intracellular PCB 153, which is shown in Figure 3.12.

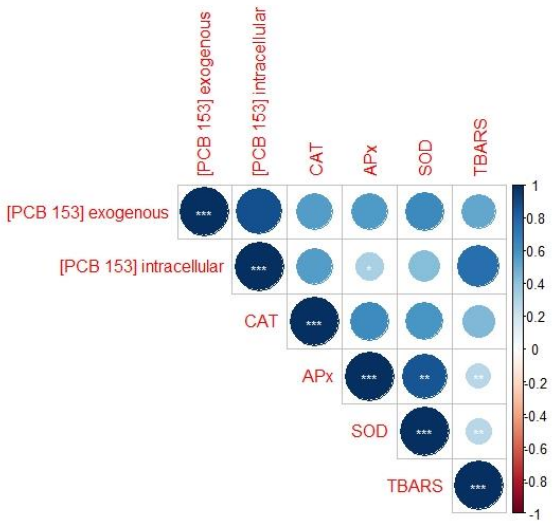


*Figure 3.12- Correlation between the catalase enzyme (CAT) (A), ascorbate peroxidase (APx) (B), superoxide oxidase (SOD), thiobarbituric acid (TBARS) with the concentrations of PCB 153 that are presented exogenous and intracellular in Phaeodactylum tricornutum following a 48 h exposure to different concentrations of PCB 153 (average ± standard error, n=3, different letters signify a significant difference of p<0.05). The darker the colour, and the bigger the dot the stronger is the correlation. When there is a * on a dot, It implies a statistical significance of p<0.05, ** p<0.01 and p<0.001.*

3.4. Discussion

The PCB 153 cell concentration increased with the concentration of this compound in the culture. Due to the lipophilic characteristics of this compound, PCB 153 can be accumulated in the lipophilic membrane of the cells [14], and thus be accumulated in diatom cells.

In the present work, it was possible to notice a significant decrease in cell density, noticed in the significant decrease in growth rate and divisions per day, with the increasing PCB 153 exogenous concentration, leading to a consequent increase in the doubling time and a growth inhibition. There are scarce works that study the effects of this type of compound and observe its toxic effects on a diatom-model species, such as the one employed in the current work. One example of such work is the one made by Mayer *et al*, (1998), which exposed the compound PCB 31 and 105 for 48h at concentrations of 900 and 27 µg/L, respectively, to the microalgae *Selenastrum capricornutum*. Even though the previous work presented a different compound, concentrations, method, and diatom from the ones used in the current work, the effect was similar to the one presented in this work: an inhibition of the growth of the microalgae occurred. In the previous work, it was stated that the reason was that a narcosis mechanism occurred and inhibited the growth of the cell [94]. This decrease in cell growth can be attributed to the PCB effects on the photosynthesis activity due to the oxidative stress caused by the contaminant.

This photosynthesis activity could be studied through the study of photochemistry and the analysis of differences in pigments concentrations that could be altered when exposed to PCB 153. As it was shown previously, the photochemistry parameters such as the net rate of PSII RC closure (Mo), the energy to close all reaction centres (S_M), reaction centre turnover rate (N), the number of available reactions centres (RC/CS), and reaction centre II density within the antenna chlorophyll bed of PSII were diminished with the increment of the PCB 153 concentration. These results point to the fact that the reaction centres suffered a toxic effect in which they were less available. This has a negative effect on the photosynthesis activity since the reaction centres are the site of the chloroplasts of the diatom where the main reactions of the photosynthesis procedure occur.

The procedure of grouping probability, which is the mechanism where energy is transferred from a closed PSII reaction centre to an open PSII reaction centre, had an increment with the increase of the concentration of PCB 153 in the cell. This procedure counteracts the unavailability of the reaction centre that was mentioned previously. So, with the increase of the reaction centre, this phenomenon occurs with a higher frequency. Regarding the decrease of grouping probability between the cultures that were exposed to 3 and 6 µg/L of PCB 153, it could be said that even though there were more closed reaction centres, there was an inhibition of this procedure.

The electron chain was also affected by the exposure of PCB 153. This could be observed by the diminishing of photochemical parameters such as the size of the quinone pool, and electron transport energy flux per cross-section (ET/CS). This inhibition of the electron chain within the diatom has a negative effect, since without the constant transport of electrons the photosynthesis activity will occur at a slower pace, affecting the health of the cell.

Furthermore, parameters such as the absorbed energy flux per cross-section (ABS/CS), trapped energy per cross-section (TR/CS), and dissipated energy flux per cross-section (DI/CS), indicate that there is a loss of energy, by not absorbing, not trapping the energy or by the dissipation of energy per cross-section of the photosynthesis mechanism when there is an incrementation of the concentration of the PCB 153. This loss of energy translates into an inhibition of this process, where the cell will not produce as much energy as the cells that were not exposed to this compound. This decrease in energy could also translate into an increase in oxidative stress in the photosystem [70].

In addition to these effects, there was also an incrementation in electron transport from PQH2 to the reduction of PSI and electron acceptors (RE_0/RC) and the efficiency of the transfer of an electron from PQH2 to the final PSI acceptors (δR_0), when there was a higher concentration of PCB 153. Since the PS I received less energy, these two results translate into a counterreaction of

the cell, where the cell received less energy but the efficiency of the electron transport from PQH2 to the PS I increased.

Finally, we could also observe a diminished structure functional index for photosynthesis (SFI) and the incrementation of the non-photochemical quenching or dissipation structure functional index, with the increase of the concentration of the analysed compound. These two translate that the structure where the photosynthesis activity occurs suffers damage, influencing it negatively.

However, even with this diminishing of the photosynthesis activity, there was an incrementation in the chlorophyll *a*, chlorophyll *c*, pheo *a*, Fucoxanthin, diadinoxanthin and diatoxanthin. This result agrees with works such as the ones made by Carvalho, R. *et al.* (2020) where the exposure of the herbicide glyphosate was studied at different concentrations (0, 10, 50, 100, 250 and 500 µg/L) to *Phaeodactylum tricornutum*. In this work, this phenomenon is described to be a counterreaction of the reaction centres of the photosystem I and II by having the ability to trap photons for reduction. The diatom-model that was employed in this work contains a PS II light-harvesting complex composition of fucoxanthin-chlorophyll protein (FCP). This protein can increase the number of reaction centres available and trap protons for a reduction since it has a mechanism where it can alter the capacity to harvest the light it receives [70].

The increase of photoprotective pigments, such as the pheo *a*, diadinoxanthin, and diatoxanthin, raises the energy dissipation by non-photochemical mechanisms, counteracting processes that could even further inhibit the photosynthesis activity [94].

Regarding the antioxidant enzymes, it was possible to notice a significant change in the activity of the three enzymes that were analysed in the current work, with the exposure of PCB 153. These results are possibly due to the increase of oxidative stress originating from the exposure to the compound in question. As of today, there are scarce works that study the response of enzymes of diatoms to these compounds, but works like the ones by Leitão, *et al.*, (2003), which used Arochlor 1254, a mixture of PCBs congeners, showed similar results to the ones presented in this work, and also concluded that these results are derived from the increase of oxidative stress provoked by the exposed compound [79]. This proves that the exposition of PCB 153 increases the creation of ROS and consecutively increases oxidative stress.

3.5. Conclusions

In conclusion, it was possible to observe that PCB 153, particularly at high concentrations, had noticeable effects on the multiple biomarkers analysed in this work (growth, photochemistry, pigments and enzyme activity). Even though several mechanisms occurred to counteract this oxidative caused by the exposition of the studied compound, such as the incrementation of grouping probability phenomenon, increase of photoprotective pigments and the increase of antioxidant mechanism, an inhibition of several photosynthesis events, and consecutively of its growth, still occurred. Since this pollutant it is lipophilic, is uptaken easily by the cells and stored in the lipophilic membrane, which could enhance these same effects in the long term.

With these results in mind, exposure to PCB 153 has negative implications for marine oxygenation, and carbon harvesting and could affect the upper levels of marine food webs, through its lipophilic characteristics and long half-life. The current work has not studied the combination of the concentration of this compound with the occurrence of other pollutants that can extend the negative impacts, beyond the ones observed in the current work.

4. General Conclusions

In the current work, it was possible to observe that even though organochloride pesticides and PCBs were banned around the 1950's, they are still present in Portuguese salt marshes, in concentrations that surpassed those suggested by the SQGs.

The concentrations of the compounds presented in the sediments represent the history and characteristics of the marsh where they were sampled. On one hand, since Rosário is a zone with higher agricultural activity in which different types of pesticides are used, it presented higher values of pesticides, which were used to protect crops against multiple types of plagues. On the other hand, since Seixal was a site with a higher industrial activity that used PCBs, it presented higher values of PCBs. Alcochete contamination values were lower for the analysed compounds than both marshes. This has a direct correlation with the characteristics of the marsh, which is more recent and upstream than the other marshes analysed in this work.

When comparing the pollutants concentrations obtained in the analysis of the sediments' marsh, it was possible to observe that the Tagus estuary presented higher values of concentration than the ones obtained in other Portuguese estuaries. This is due to the higher population and consecutively, higher anthropogenic activity than the other Portuguese estuaries.

Concerning the pollutants concentrations on plants, it was possible to observe that there was a transport of certain compounds from belowground to aboveground.

Concerning the ecotoxicological texts, where the organochloride compound PCB 153 was exposed to the diatom *Phaeodactylum tricornutum*, it was possible to observe, particularly at high concentrations, notorious effects on the biomarkers analysed in this work (growth, photochemistry, pigments and enzyme activity). Despite several mechanisms to counteract this oxidative stress, like the incrementation of grouping probability phenomenon increase of photoprotective pigments and the increase of antioxidant mechanism, negative effects could still be observed. There was an inhibition of several photosynthesis events and cell growth. Lipophilic compounds such as PCB 153, are uptake efficiently by the cells and stored in the lipophilic membrane, which in the long term could aggravate the effects.

By observing the results obtained in the current work, it was possible to state that the exposure to PCB 153 brings negatives effects for the marine oxygenation, carbon harvesting and upper levels of marine food webs, enhancing the effects through its lipophilic characteristics and extensive half-life.

In future work, the combination of other pollutants present in Tagus marsh could be studied, such as metal concentration, microplastics, and other organochloride compounds that were not studied in the current work.

5. Bibliography

[1]. Hoffman, D. J.; Rattner, B., Burton G. A. and Cairns, J., 1995. *Handbook of Ecotoxicology*. Lewis Publishers, CRC press London.

[2]. Gadd, G. M., 1990. *Accumulation of metals by microorganisms and algae*. Experientia 46**,** 834–840. https://doi.org/10.1007/BF01935534.

[3]. Webster, L., Roose, P., *et al.*, 2013. *Determination of polychlorinated biphenyls (PCBs) in sediments and biota.* ICES Techniques in Marine Enviromental Sciences Nº53. https://doi.org/10.17895/ices.pub.5078.

[4]. Nicolopoulou-stamati, P., 2016. *Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture*. *4*(July), 1–8. https://doi.org/10.3389/fpubh.2016.00148.

[5]. Casida, J.E., Quistad, G.B.., 1998. *Golden Age of Insecticide Research: Past, Present, or Future?*. Annual Reviews Entomology. 43: 1-16. doi: 10.1146/annurev.ento.43.1.1.

[7]. Barbara, S., 2012. *Determinação de Resíduos de Pesticidas em produtos de origem vegetal por GC-ECD*. Tese de mestrado, Universidade de Lisboa, Faculdade de Ciências. http://hdl.handle.net/10451/7448.

[8]. Pimentel, R., 2007. *Desenvolvimento de Metodologias Analíticas para a Determinação de Resíduos de Pesticidas em Medicamentos à Base de Plantas*. Tese de mestrado, Universidade de Lisboa, Faculdade de Farmácia. http://hdl.handle.net/10451/241.

[9]. Harada, T., Takeda, M., Kojima, S., and Tomiyama, N., 2016. *Toxicity and Carcinogenicity of Dichlorodiphenyltrichloroethane (DDT)*. Toxicol Res, *32*(1), 21–33. doi: 10.5487/TR.2016.32.1.021.

[10]. Faroon, O., Ruiz, P., *et al.*, (2019). *Toxicological Profile for DDT, DDE, and DDD*. U.S. Department of Health and human services.

[11]. Ghosh, S., De, S., Chen, Y. *et al.*, 2020. *Polychlorinated biphenyls (PCB-153) and (PCB-77) absorption in human liver (HepG2) and kidney (HK2) cells in vitro: PCB levels and cell death*. Environmental International 36, 893-900. https://doi:10.1016/j.envint.2010.06.010.

[12]. Nakari, T., Huhtala, S., 2008. *Comparison of toxicity of congener-153 of PCB, PBB, and PBDE to Daphnia magna*. Ecotoxicology and Environmental Safety 71, 514–518. doi:10.1016/j.ecoenv.2007.10.012.

[13]. National Institute of Health, 2006. *TOXICOLOGY AND CARCINOGENESIS STUDIES OF 2,2N,4,4N,5,5N-HEXACHLOROBIPHENYL (PCB 153)*. Public Health Service U.S. Department of Health and Human Services. https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr529.pdf.

[14]. Agency for Toxic Substances and Disease Registry (ATSDR), 2014. *Toxicological Profile for Polychlorinated Biphenyls*. U.S. Department of Health and Human Services, Public Health Service Atalanta, GA.

[15]. Carpenter, D. O., 2006. *Polychlorinated Biphenyls (PCBs): Routes of Exposure and Effects on Human Health*. Reviews on Environmental Health, Volume 21(1):1-23. doi: 10.1515/reveh.2006.21.1.1.

[16]. Rom, W. N., Markowitz, S B., 2007. *Environmental and Occupational Medicine*. 4th ed. Rom, W.N. editor. Chapter 77: Polychlorinated Biphenyls. Lippincott Williams & Wilkins.

[17]. Talley, L. D., Pickard, G. L., Emery, W. J., and Swift J. H., 2011. *Descriptive Physical Oceanography: An Introduction*. Elsevier, Boston, 6th edition, 560 pp.

[18]. Sekerci, Y., and Petrovskii, S., 2015. *Mathematical Modelling of Plankton–Oxygen Dynamics Under the Climate Change*. Bulletin of Mathematical Biology, 77(12), 2325–2353. https://doi.org/10.1007/s11538-015-0126-0.

[19]. Cardoso, M. N., 2013. *Persistent Organic Pollutants in Portuguese Estuaries: Occurrence and distribution of PCDD/Fs and dioxin-like PCBs*. Tese de mestrado, Faculdade de Ciências e Tecnologia da Universidade de Coimbra. hdl.handle.net/10316/24274.

[20]. Torre, C., 2014. *Hidrocarbonetos Aromáticos Policíclicos: Concentração e Biodisponibilidade*. Tese de Mestrado, Faculdade de Ciências, Universidade de Lisboa. http://hdl.handle.net/10451/15701.

[21]. Gonçalves, P., 2016. *Os sapais em Portugal Continental Levantamento e evolução das suas envolventes desde 1990 a 2012*. Tese de Mestrado, Instituto Superior de Agronomia da Universidade de Lisboa. http://hdl.handle.net/10400.5/13849.

[22]. Teixeira, A., Duarte, B. and Caçador, I., 2014. *Salt marshes and Biodiversity*. Tasks for Vegetation Science Vol. 47. Sabkha Ecosystems: Volume IV: Cash Crop Halophytes and Biodiversity Conservation. doi:10.1007/978-94-007-7411-7_20.

[23]. Neto, J. M.; Caçador, I.; Caetano, M.; *et al*., 2019. *Rios de Portugal: comunidades, processos e alterações*. Coimbra University press, cap. 16, pags. 382-421. https://doi.org/10.14195/978-989-26-1624-7_16.

[24]. Reis-Santos, P., Pais, M., Duarte, B., Caçador, I., *et al.*, 2018. *Screening of human and veterinary pharmaceuticals in estuarine waters: a baseline assessment for the Tejo estuary*. Marine Pollution Bulletin 135, 1079-1084. doi:10.1016/j.marpolbul.2018.08.036

[25]. Duarte, B., Reboreda, R., Cacador, I., 2008. *Seasonal variation of Extracellular Enzymatic Activity (EEA) and its influence on metal speciation in a polluted salt marsh*. Chemosphere 73, 1056–1063. https://doi.org/10.1016/j.chemosphere.2008.07.072.

[26]. Bettencourt, A.M.M., Andreae, M.O., Cais, Y., Gomes, M.L., Schebek, L., 1999. *Organotin in the Tagus estuary*. Aquatic Ecology 33, 271–280. https://doi.org/10.1023/A:1009966006325.

[27]. Canário, J., Vale, C., Caetano, M., 2005. *Distribution of monomethylmercury and mercury in surface sediments of the Tagus Estuary (Portugal)*. Marine Pollution Bulletin 50, 1142–1145. doi: 10.1016/j.marpolbul.2005.06.052.

[28]. Cottè-Krief, M.H., Guieu, C., Thomas, A.J., Martin, J.M., 2000. *Sources of Cd, Cu, Ni and Zn in Portuguese coastal waters*. Marine Chemistry 71, 199–214. https://doi.org/10.1016/S0304-4203(00)00049-9.

[29]. Vale, C., 1990. *Temporal variations of particulate metals in the Tagus River Estuary*. Science of Total Environment 97–98, 137–154. https://doi.org/10.1016/0048-9697(90)90236-N.

[30]. Duarte, B., Santos, D., Marques, J.C., Caçador, I., 2013a. *Ecophysiological adaptations of two halophytes to salt stress: photosynthesis, PS II photochemistry and antioxidant feedback e implications for resilience in climate change*. Plant Physiology Biochemistry 67, 178-188. doi: 10.1016/j.plaphy.2013.03.004.

[31]. Duarte, B., Santos, D., Caçador, I., 2013b. *Halophyte anti-oxidant feedback seasonality in two salt marshes with different degrees of metal contamination: search for an efficient biomarker*. Functional Plant Biology 40, 922-930. doi:10.1071/FP12315.

[32]. Duarte, B., Santos, D., Marques, J.C., Caçador, I., 2014. *Biophysical probing of Spartina maritima photo-system II changes during increased submersion periods: possible adaptation to sea-level rise*. Plant Physiology Biochemistry 77, 122-132. https://doi.org/10.1016/j.plaphy.2014.01.023.

[33]. Duarte, B., Marques, J.C., Caçador, I., 2015. *Impact of extreme heat and cold events on the energetic metabolism of the C3 halophyte Halimione portulacoides*. Estuarine Coastal and Shelf Science 167, 166-177. https://doi.org/10.1016/j.ecss.2015.10.003.

[34]. Duarte, B., Caçador, I., Marques, J.C. and Croudace, I., 2013. *Tagus Estuary salt marshes feedback to sea level rise over a 40-year period: insights from the application of geochemical indices*. Ecological Indicators 34, 268-276. doi:10.1016/j.ecolind.2013.05.015.

[35]. Pedro, S., Duarte, B., Almeira, P.R. and Caçador, I., 2015. *Metal speciation in salt marsh sediments: influence of halophyte vegetation in salt marshes with different morphology*. Estuarine Coastal and Shelf Science 167, 248-255. https://doi.org/10.1016/j.ecss.2015.05.034.

[36]. Duarte, B., Caetano, M., Almeida, P., Vale, C. and Caçador, I., 2010. *Accumulation and biological cycling of heavy metal in the root-sediment system of four salt marsh species, from Tagus estuary (Portugal)*. Environmental Pollution 158, 1661-1668. doi:10.1016/j.envpol.2009.12.004.

[37]. Caçador, I., Neto, J.M., Duarte, B., Barroso, D.V., Pinto, M. and Marques, J.C., 2013. *Development of an Angiosperm Quality Assessment Index (AQuA – Index) for ecological quality evaluation of Portuguese water bodies – A multi-metric approach*. Ecological Indicators 25, 141-148. doi:10.1016/j.ecolind.2012.09.021.

[38]. Marchant, C., Goodman, P., 1969. *Spartina maritima (Curtis) Fernald*. J Ecol 57:287–291. https://doi.org/10.2307/2258222.

[39]. Redondo-Gómez, S., 2012. *Bioaccumulation of heavy metals in Spartina*. Functional Plant Biology 40(9) 913-921. doi: 10.1071/FP12271.

[40]. J., Ravindran, M., Pankajshan, S., Puthur. 2017. *Review Article. Organochloride pesticides, their toxic effects on living organisms and their fate in the environment*. Interdiscip Toxicol. 2016; Vol. 9(3–4): 90–100. doi: 10.1515/intox-2016-0012.

[41]. Helfrich, L., Hipkinsm, P., 2009. *Pesticides and Aquatic Animals: A Guide to Reducing Impacts on Aquatic Systems*. Virginia Polythecnic Institute and State University. https://pubs.ext.vt.edu/420/420-013/420-013.html.

[42]. Barceló, D., 2000. *Sample handling and Trace Analysis of Pollutants*. Elsevier, Ed 1.

[43]. European Comission, 2019.

[44]. Ospar,2010. QUality Satus Report 2020. Ospar Comission.

[45]. Wisconsin Department of Natural Resources,2003. Consensus-Based Sediment Quality Guidelines. Wisconsin Department of Natural Resources.

[46]. NT.LB.22 v.01.02 (2020-11-19) – *Análise granulométrica – Método de Peneiração*.

[47]. NT.LB.23 v.02.02 (2015-03-13) – *Análise granulométrica – Método de Difração Laser*.

[48]. NT.LB.26 – *Determinação de Carbono Orgânico Total em Sedimentos.*

[49]. EN 12880:2000. *Characterization of sludges- Determination of dry residue and water content.* European Committee for Standardization. 9 pp.

[50]. EN 15169:2007. *Characterization of waste- Determination of loss on ignition in waste, sludge and sediments.* European Committee for Standardization. 13 pp.

[51]. Dionex, 1998. *Technical Note 208 – Methods Optimization in Accelerated Solvent Extraction (ASE).*

[52]. Dionex, 1999. *Application Note 320 – Extraction of Chlorinated Pesticides Using Accelerated Solvent Extraction (ASE).*

[53]. Dionex, 1999. *ASE 200 Accelerated Solvent Extractor Operator's Manual*, Revision 04, California, Dionex Corporation, U.S.A.

[54]. Dionex, 2004. *Application Note 316 – Extraction of PCBs from Environmental Samples Using Accelerated Solvent Extraction (ASE)*

[55]. ISO 10382:2002 (E). *Soil Quality - Determination of Organochloride Pesticides and Polychlorinated Biphenyls - Gas Chromatography Method with Electron Capture Detection.*

[56]. EPA Method 8000D. *Revision 5: Determinative Chromatographic Separations. The United States Environment Protection Agency*.

[57]. OSPAR 2002. *JAMP Guidelines for Monitoring Contaminants in Sediments*. OSPAR Commission. Monitoring guidelines, ref. no. 2002-16.

[58]. Duarte, B., Freitas, J., Couto, T., *et al*., 2013. *New multi-metric Salt Marsh Sediment Microbial Index (SSMI) application to salt marsh sediments ecological status assessment*. Ecological Indicators 390-397. https://doi.org/10.1016/j.ecolind.2013.01.008.

[59]Silva, T., 2013. *Sedimentologia e morfodinâmica de sapais do estuário do Tejo em cenários de alteração climática*. Tese de Mestrado, Universidade de Lisboa. http://hdl.handle.net/10451/10507.

[60]. Carvalho, P., Rodrigues, P., Basto, M., *et al*., 2009. *Organochloride pesticides levels in Portuguese coastal areas*. Chemosphere 75 595–600. doi:10.1016/j.chemosphere.2009.01.060.

[61]. Vale, O., 1999. *DDT concentrations in surficial sediments of three estuarine systems in Portugal*. Aquatic Ecology 33: 263-269. https://doi.org/10.1023/A:1009961901782.

[62]. Mil-Homens, M., Vicente, M., Grimalt, J., *et al*., 2016. *Reconstruction of organochloride compound inputs in the Tagus Prodelta*. Science of the Total Environment 540, 231-240. https://doi.org/10.1016/j.scitotenv.2015.07.009.

[63]. Lobo, J., Costa, P. M., Caeiro, S., *et al*., 2010. *Evaluation of the potential of the common cockle (Cerastoderma edule L.) for the ecological risk assessment of estuarine sediments: bioaccumulation and biomarkers.* Ecotoxicology 19:1496-1512. doi:10.1007/s10646-010-0535-7.

[64]. Villanueva, C.M., Kogevinas, M., Cordier, S., Templeton, M.R., Vermeulen, R., Nuckols J.R., *et al.*, 2014. *Assessing exposure and health consequences of chemicals in drinking water: Current state of knowledge and research needs*. Environmental Health Perspectives. 122(3):213-221. doi: 10.1289/ehp.1206229.

[65]. Grilo, T.F., Cardoso, P.G., Pato, P., *et al.*, 2014. *Uptake and depuration of PCB-153 in edible shrimp Palaemonetes varians and human health risk assessment*. Ecotoxicology and Environmental Safety 101, 97-102. http://dx.doi.org/10.1016/j.ecoenv.2013.12.020.

[66]. Monikh, F., Karami, O., Hosseini, M., *et al.*, 2013. The effect of primary producers of experimental aquatic food chains on mercury and PCB153 biomagnification. 94, 112-115. https://doi.org/10.1016/j.ecoenv.2013.05.013.

[67]. Arrebola, J.P., Fernandez, M.F., Martin-Olmedo, P., Molina-Molina, J.M., Sanchez-Perez, M.J., Sanchez-Cantalejo, E., *et al.*, 2014. *Adipose tissue concentrations of persistent organic pollutants and total cancer risk in an adult cohort from southern Spain: preliminary data from year 9 of the follow-up*. Sci. Total Environ. 500-501, 243–249. doi: 10.1016/j.scitotenv.2014.08.043.

[68]. Kashima, S., Yorifuji, T., Tsuda, T., Eboshida, A., 2015. *Cancer and non-cancer excess mortality resulting from mixed exposure to polychlorinated biphenyls and polychlorinated dibenzofurans from contaminated rice oil: "Yusho*". Int. Arch. Occup. Environ. Health 88, 419–430. doi: 10.1007/s00420-014-0966-1.

[69]. Livingstone DR (2001). *Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms*. Mar Pollut Bull 42(8):656–666. doi:10.1016/s0025-326x(01)00060-1.

[70]. Carvalho, R. C. De, Feijão, E., Matos, A. R., Cabrita, M. T., Duarte, B., *et al.*, 2020. *Glyphosate-Based Herbicide Toxicophenomics in Marine Diatoms: Impacts on Primary*

Production and Physiological Fitness. Appl. Sci, 10(21), 1–24. https://doi.org/10.3390/app10217391.

[71]. Feijão, E.; Gameiro, C.; Franzitta, M.; Duarte, B.; Caçador, I.; Cabrita, M.T.; Matos, A.R. *Heat wave impacts on the model diatom Phaeodactylum tricornutum: Searching for photochemical and fatty acid biomarkers of thermal stress*. Ecol. Indic. 2018, 95, 1026–1037. https://doi.org/10.1016/j.ecolind.2017.07.058.

[72]. Dodson, V.J.; Mouget, J.L.; Dahmen, J.L.; Leblond, J.D. *The long and short of it: Temperature-dependent modifications of fatty acid chain length and unsaturation in the galactolipid profiles of the diatoms Haslea ostrearia and Phaeodactylum tricornutum*. Hydrobiologia 2014, 727, 95–107. doi:0.1007/s10750-013-1790-4.

[73]. Cabrita, M.T.; Duarte, B.; Gameiro, C.; Godinho, R.M.; Caçador, I. *Photochemical features and trace element substituted chlorophylls as early detection biomarkers of metal exposure in the model diatom Phaeodactylum tricornutum*. Ecol. Indic. 2018, 95, 1038–1052. doi:10.1016/j.ecolind.2017.07.057.

[74]. Abida, H.; Dolch, L.-J.J.; Meï, C.; Villanova, V.; Conte, M.; Block, M.A.; Finazzi, G.; Bastien, O.; Tirichine, L.; Bowler, C.; *et al*., 2015. *Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in Phaeodactylum tricornutum*. Plant Physiol. 167, 118–136. https://doi.org/10.1104/pp.114.252395.

[75]. Liu, Q.; Tang, X.; Wang, Y.; Yang, Y.; Zhang, W.; Zhao, Y.; Zhang, X. 2019. *ROS changes are responsible for tributyl phosphate (TBP)-induced toxicity in the alga Phaeodactylum tricornutum*. Aquat. Toxicol. 208, 168–178. doi: 10.1016/j.aquatox.2019.01.01.

[76]. Cabrita, M.T.; Duarte, B.; Gameiro, C.; Godinho, R.M.; Caçador, I. 2018. *Photochemical features and trace element substituted chlorophylls as early detection biomarkers of metal exposure in the model diatom Phaeodactylum tricornutum*. Ecol. Indic. 95, 1038–1052. https://doi.org/10.1016/j.ecolind.2017.07.057.

[77]. Cabrita, M.T.; Raimundo, J.; Pereira, P.; Vale, C. 2014. *Immobilised Phaeodactylum tricornutum as biomonitor of trace element availability in the water column during dredging*. Environ. Sci. Pollut. Res. 21, 3572–3581. doi:10.1007/s11356-013-2362-x.

[78]. Leitão, M. A., Cardozo, K. H. M., Pinto, E., and Colepicolo, P., 2003. *PCB-Induced Oxidative Stress in the Unicellular Marine Dinoflagellate Lingulodinium polyedrum*. Arch. Environ. Contam. Toxicol. 45, 59–65. doi:10.1007/s00244-002-0208-5.

[79]. Wang, Y., Mu, W., Sun X., *et al.*, 2020. *Physiological response and removal ability of freshwater diatom Nitzschia palea to two organophosphorus pesticides*. Chemistry and Ecology, 36:9, 881 902, doi:10.1080/02757540.2020.1784883.

[80]. Anjum, N. A., Duarte, B., Caçador, I., and Sleimi, N., 2016. *Biophysical and Biochemical Markers of Metal / Metalloid-Impacts in Salt Marsh Halophytes and Their Implications*. Frontiers in Environmental Science, 4, 1–13. https://doi.org/10.3389/fenvs.2016.00024.

[81]. Gao, J., Wang, H.; Yuan, Q., 2020. *Structure and Function of the Photosystem Supercomplexes*. Frotiers in plant science, volume 9, article 357 doi: 10.3389/fpls.2018.00357.

[82]. Duarte, B., Durante, L., Marques, J.C., Reis-Santos, P., Fonseca, V.F. and Caçador, I., 2021. *Development of a toxicophenomic index for trace element ecotoxicity tests using the halophyte Juncus acutus: Juncus-TOX*. Ecological Indicators 121, 107097. DOI: 10.1016/j.ecolind.2020.107097.

[83]. Duarte, B., Carreiras, J., Feijão, E., Reis-Santos, P., Caçador, I., Matos, A.R. and Fonseca, V.F., 2021. *Fatty acid profiles of estuarine macroalgae are biomarkers of*

anthropogenic pressures: development and application of a multivariate pressure index. Science of the Total Environment 788, 147817. doi:10.1016/j.scitotenv.2021.147817.

[84]. Feijão, E., Carvalho, E., Duarte, I., Duarte, B., *et al*. 2020. *Fluoxetine Arrests Growth of the Model Diatom Phaeodactulum tricornutum by Increasing Oxidative Stress and Altering Energetic and Lipid Metabolism*. Front. Microbiol. 11:1803. doi:10.3389/fmicb.2020.01803

[85]. Feijão, E. 2017. *Consequences of Global Warming and Metal Pollution on the metabolism of omega-3 and -6 fatty acids of Phaeodactylum tricornutum.* Tese de mestrado, Universidade de Lisboa, Faculdade de Ciências. http://hdl.handle.net/10451/30777.

[86]. OECD, 2002. *Organisation for Economic Co-operation and Development, Freshwater algal and cyanobacteria, growth inhibition test – test guideline 201, 2002*. OECD Guidelines for the testing of chemicals, Paris, France, vol. 1 (2), pp. 1–25.

[87]. Küpper, H., Seibert, S., Parameswaran, A., 2007. *Fast, sensitive, and inexpensive alternative to analytical pigment HPLC: Quantification of chlorophylls and carotenoids in crude extracts by fitting with Gauss peak spectra*. Anal. Chem. 79, 7611–7627. doi:10.1021/ac070236m.

[88]. Heath, R. L., and Packer, L. 1968. *Photoperoxidation in isolated chloroplasts*. Arch. Biochem. Biophys. 125, 189–198. doi: 10.1016/0003-9861(68)90654-1.

[89]. Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. doi: 10.1016/0003-2697(76)90527-3.

[90]. Teranishi, Y.; Tanaka, A.; Osumi, M.; Fukui, S. 1974. *Catalase activities of hydrocarbon-utilizing Candida yeast*. Agric. Biol. Chem. 1974, 38, 1213–1220. https://doi.org/10.1080/00021369.1974.10861301.

[91]. Tiryakioglu, M.; Eker, S.; Ozkutlu, F.; Husted, S.; Cakmak, I. 2006. *Antioxidant defense system and cadmium uptake in barley genotypes differing in cadmium tolerance*. J. Trace Elem. Med. Biol. 2006, 20, 181–189. doi: 10.1016/j.jtemb.2005.12.004.

[92]. Marklund, S.; Marklund, G. 1974. *Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase*. Eur. J. Biochem. 47, 464–469. doi: 10.1111/j.1432-1033.1974.tb03714.x.

[93]. Mayer, P., Nyholm, N., *et al.*, 1998. *Toxic cell concentrations of three polychlorinated biphenyl congeners in the green alga Selenastrum capricornutum.* Environmental Toxicology and Chemistry, Vol. 17, No. 9, pp. 1848–1851. https://doi.org/10.1002/etc.5620170927.

[94]. Duarte, B.; Pedro, S.; Marques, J.C.; Adão, H.; Caçador, I. 2017. *Zostera noltii development probing using chlorophyll a transient analysis (JIP-test) under field conditions: Integrating physiological insights into a photochemical stress index*. Ecol. Indic. 76, 219–229, doi:10.1016/j.ecolind.2017.01.023.