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Pesticides in water, sediments and biota of semi-closed coastal lagoons: sources, pathways and impact on aquatic organisms

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In memory of my Parents...

You should go on learning for as long as your ignorance lasts;

and, if the proverb is to be believed,

for the whole of your life.

Lucius Annaeus Seneca

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Abstract

Pesticides are vast class of compounds formulated to control pest. They are applied all over the world and their presence in the environment, especially in coastal lagoons has been inevitable. The knowledge gap concerning pesticides fate in coastal lagoons and their impact on the aquatic organisms as well as the lack of sensible analytical methods for their measurement was the driving force of this work. Focus was made on the pesticides classified as priority substances within the Water Framework Directive (WFD). Óbidos Lagoon (Leiria, Portugal) is of economic importance and as any coastal lagoon, is exposed to anthropogenic activities being for those reason the selected area for all the studies.

To fulfill the analytical gaps concerning the priority pesticides (PPs), sensible analytical methodologies were developed for the determination of those pesticides in sediments and macroalgae *Ulva* sp. of Óbidos Lagoon. The application of such methodologies allowed an in-depth knowledge of pesticides historical application, sources and pathways inside the lagoon. Interestingly, results in the analysis of *Ulva* sp. show a tendency to accumulate some of the PPs only under adverse weather conditions which points the importance of the climate global changes in the uptake and partition of the PPs in coastal lagoons. Rural activities in the watershed were found to be the main source of the PPs in

Óbidos Lagoon. Soil runoff and discharges through small tributaries are the main vehicles of entrance into the lagoon. PPs sediments monitoring reveals that past and present applications of those compounds have been carried out. The low hydrodynamism of the lagoon branches (*Barrosa*) favors the retention of the pesticides in this part of the lagoon. Among the list of the studied PPs, lindane, *p,p'*-DDT and the metabolite heptachlor epoxide show to be at levels above the ''probable level effect'' with possible adverse impacts in aquatic organisms. Dissipation studies with chlorpyrifos revealed the importance of salinity, water turbulence and addition of dissolved organic matter (DOM) in its environmental fate. DOM is a very complex mixture of thousands of organic compounds with different sizes, charges and polarity. The development of a new gradient elution extraction methodology that uses a mixture of eluents with different polarities allowed the fractionation of DOM based in its hydrophobicity. Improving knowledge of DOM molecular-level composition is crucial for a better understanding of its reactivity and consequently its impact on pesticides environmental fate.

Keywords: Pesticides; Priority substances; Analytical methodologies; WFD; Sediments; Fate; Macroalgae; DOM.

Resumo

Os pesticidas são uma vasta classe de compostos formulados para o controle de pragas. Eles são aplicados mundialmente e a sua presença no ambiente, especialmente em lagoas costeiras tem sido inevitável. A lacuna existente sobre o destino dos pesticidas nas lagoas costeiras e seu impacto nos organismos aquaticos, assim como a falta de métodos analíticos sensíveis para a sua determinação foi a força motriz deste trabalho. Foi dada enfase aos pesticidas classificados como substâncias prioritárias no âmbito da Directiva-Quadro da Água. Foi selecionada a Lagoa de Óbidos dada a sua biodiversidade e importância económica, e tal como a maior parte das lagoas costeiras na Europa, está exposta a atividades antropogénicas.

De modo a suprimir as lacunas analíticas relativas aos pesticidas prioritários (PPs) foram desenvolvidos metodologias analíticas para a determinação de pesticidas em sedimentos e em macroalgas *Ulva* sp. da Lagoa de Óbidos. Da aplicação das metodologias desenvolvidas resultou um conhecimento mais aprofundado do histórico de contaminação, proveniência e transporte dentro da lagoa. Interessantes resultados foram encontrados na análise de *Ulva* sp.. Esta macroalga mostrou uma tendência para acumular alguns dos PPs apenas sob condições metereológicas adversas o que mostra a importância das mudanças climáticas globais no futuro da distribuição de PPs

em lagoas costeiras, designadamente nos processos de absorção e repartição destes compostos no ambiente. Os resultados mostraram que a actividades rurais na bacia de drenagem são a principal fonte dos PPs na lagoa de Óbidos. O escoamento superficial e a descarga de pequenos afluentes constituem o principal veículo para a entrada dos pesticidas na lagoa. A monitorização dos PPs em sedimentos revelou aplicações anteriores e presentes destes compostos. Os resultados mostraram, ainda, que o baixo hidrodinamismo dos braços da lagoa (*Barrosa)* favorece a retenção dos PPs nesta zona. Entre os PPs estudados, o lindano, *p,p'*-DDT e o metabolito heptacloro epóxido apresentaram níveis acima do "niveís com efeitos prováveis" com potenciais impactos adversos em organismos aquáticos. Os estudos de dissipação com o clorpirifos revelaram a importância da salinidade, da turbulência da água e da adição de matéria orgânica dissolvida (*DOM*) na repartição do insecticida no ambiente. A matéria orgânica dissolvida é uma mistura complexa constituída por um grande número de compostos orgânicos com diferentes tamanhos, cargas e polaridades. O desenvolvimento de um nova metodologia de extracção baseada na eluição dos composto em forma de gradiente usando uma mistura de eluentes com diferentes polaridades permitiu o fraccionamento da *DOM* com base nas suas características hidrofóbicas. O conhecimento da composição da *DOM* a nível molecular é crucial para uma melhor previsão da sua reactividade, e consequentemente, do seu impacto na distribuição dos pesticidas no ambiental.

Palavras-chave: Pesticidas, Subtâncias prioritárias; Metodologias analíticas Directiva-Quadro da Água; Sedimentos; Distribuição ambiental; Macroalgas; *DOM*.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

A	Absorbance
a (m^{-1})	Absorption coefficient
E2:E3	Absorbance ratio A_{250nm}/A_{365nm}
eV	Electron Volt
H	Henry's Law Constant
Hg	Mercury
k	Photodegradation rate constant
k_{ow}	Octanol-water partitioning coefficient
m/z	Mass-to-charge ratio
$^{\bullet}OH$	Hydroxyl radical
$^1O_2$	Singlet oxygen
r^2	Correlation coefficient
S	Spectra slope
S_R	Spectral slope ratio ($S_{275\text{-}295\ nm}/S_{350\text{-}400\ nm}$)
S/N	Signal-to-noise ratio
$t_{1/2}$	Half-life
λ	Wavelength (nm)

Abbreviations

AA	Annual average concentration
AAS	Atomic absorption spectroscopy
ACN	Acetonitrile
AS	Active substance
ASE	Accelerated solvent extraction
ASE Prep MAP	Dionex absorbing moisture polymer
ASTM	American National Standard Institute
BCF	Bioconcentration factor
BMF	Biomagnification factor
CAS	Chemical abstract system number

CDOM	Chromophoric dissolved organic matter
CRAM	Carboxylic-rich alicyclic molecules
CRM	Certified reference materials
DB-5	GC capillar column -5% phenyl, 95% dimethylpolysiloxane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DE	Diatomaceous earth
DEHPA	Dichloromethane, di(2-ethylhexyl)phosphoric acid
DHF-LPME	Dynamic hollow-fiber liquid-phase microextraction
DHS-THE-LPME	Dynamic headspace time-extended helix liquid-phase microextraction
DHT-LPME	Dynamic hook-type liquid-phase microextraction
DLLME	Dispersive liquid-liquid microextraction
DLLME-LSC	Dispersive liquid-liquid microextraction with little solvent consumptions
DLLME-SFO	Solidification of a floating organic drop
DMDD	Dimethoxydiphenyldichloroethane
DMDE	1,1-dichloro-2,2-di(4-methoxyphenyl)ethene
DMSO	Dimethyl sulfoxide
DOM	Dissolved organic matter
dw	Dry weight
EC	European Community
EEMs	Excitation emission matrix fluorescence spectroscopy
EI	Electron Impact ionization
Em	Wavelength of Emission
EN	European Committee for Standardization
EPA	Environmental Protection Agency
EQS	Environmental quality standards
EU	European Union
Ex	Wavelength of Excitation
FID	Flame ionization detector
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
FT-IR	Fourier transform infrared radiation

GCB	Graphitized carbon black
GC-ECD	Gas chromatography-electron capture detection
GC-FID	Gas chromatography-flame ionization detection
GC-FPD	Gas chromatography-flame photometric detection
GC×GC	Two-dimensional gas chromatography
GC-HRMS	Gas chromatography-high resolution mass spectrometry
GC-μEC	Gas chromatography-μelectron capture detection
GC-NCI-MS	Gas chromatography-negative chemical ionization-mass spectrometry
GC-NPD	Gas chromatography-nitrogen phosphorous detection
GC-MS	Gas chromatography-mass spectrometry
HCBD	Hexachlorobutadiene
HCH	Hexachlorocyclohexane
HF-LPME	Hollow-fiber liquid-phase microextraction
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography diode array detection
HPLC-FLD	High performance liquid chromatography fluorescence detection
HPLC-UV	High performance liquid chromatography Ultraviolet detection
HP-5MS	GC capillar column -5% phenyl, 95% dimethylpolysiloxane
HS-SDME	Headspace single-drop microextraction
IEC	International Electrotechnical Commission
IL	Ionic liquids
IHSS	International Humic Substances Society
IL-DLLME	Ionic liquid dispersive liquid-liquid microextraction
IRWD	Irvine Range Water District
IS	Internal standard
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LC-UV	Liquid chromatography coupled to UV spectroscopy
LD_{50}	Lethal dose, 50%

LLE	Liquid-liquid extraction
LLSME	Liquid-liquid-solid microextraction
LP Hg lamp	Low-pressure mercury lamp
LPME	Liquid-phase microextraction
LOD	Limit of detection
LOQ	Limit of quantification
LSC	Little solvent consumptions
LVI	Large volume injection
MASE	Membrane-assisted solvent extraction
MAC	Maximum allowable concentration
MAE	Microwave-assisted extraction
MDL	Method detection limit
MDTL	Material derived from linear terpenoids
ME	Matrix effect
MeOH	Methanol
MIP	Molecular imprinted polymer
MLPME	Membrane liquid-phase microextraction
MMLLE	Microporous-membrane liquid/liquid extraction
MP Hg lamp	Medium-pressure mercury lamp
^{3}NOM*	Triplet state excited natural organic matter
MQL	Method quantification limit
MRL	Maximum residue level
MSD	Mass spectrometry detector
MTBSTFA	*N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide
MW	Molecular weight
n.a	Not applicable
n.d	Not detected
NPBB	Newport Back Bay
NMR	Nuclear magnetic resonance
OL	Óbidos Lagoon
PDMS	Polydimethylsiloxane
PEL	Probable effect level
PHS	Priority hazardous substance
PLE	Pressurized liquid extraction

PPL I	SPE methanol:water extract (1:1, v:v)
PPL II	SPE methanol:acetonitrile:water extract (1:1:2, v:v:v)
PPL III	SPE methanol:acetonitrile extract (1:1, v:v)
POPs	Persistent organic pollutants
PPs	Priority pesticides
PPPs	Plant protection products
PS	Priority substances
QSU	Quinine sulphate units
RCF	Relative centrifugal force
RP-HPLC	Reversed phase high performance liquid chromatography
rpm	Rotation per minute
RSD	Relative standard deviation
SBSE	Stir bar sorptive extraction
SDCME	Single-drop coacervative microextraction
SDME	Single-drop microextraction
SJ IRWD	San Joaquin Irvine Water District wetlands
SFE	Supercritical fluid extraction
SFO	Solidification of a floating organic drop
SIM	Selective ion monitoring
SLME	Supported-liquid membrane extraction
SPE	Solid phase extraction
SPLE	Selective pressurized liquid extraction
SPM	Suspended particulate matter
SPME	Solid phase microextraction
SRM	Standard Reference Material
SRNOM	Suwannee River natural organic matter
XAD-4	Styrene-divinylbenzene resins
XAD-8	Acrylic resins
TBP	Tri-*n*-butyl phosphate
TCP	3,5,6-trichloro-2-pyridinol
TMP	3,5,6-trichloro-2-methoxy-pyridine
TOPO	Tri-*n*-octylphosphine oxide
UAA	Utilized agricultural area
UAE	Ultrasound-assisted extraction

UHPLC	Ultra high performance liquid chromatography
USAEME	Ultrasound-assisted emulsification-microextraction
UV	Ultraviolet
VP	Vapor pressure
WFD	Water Framework Directive
WHO	World Human Organization

1

General Introduction

1.1 Pesticides

1.1.1 Definition and classification

Pesticide is defined as any substance or mixture of substances, natural or synthetic, used to destroy, control or repel any pest [1, 2]. They are usually chemical substances, although they can be biological agents such as virus or bacteria [1-3]. Pesticides can be classified in several ways depending on the purpose they are meant to be used. Pesticides applied in the agriculture for crop protection are referred as plant protection products (PPPs) or as agrochemical pesticides while the ones applied in non-agriculture sectors such as wood and textile preservation are referred as biocides [4, 5]. Pesticides can also be organized according to their mode of action, chemical composition, use patterns (e.g., foliar *vs*. soil fungicide) or according to the group of pests to be controlled [6]. Herbicides, insecticides, fungicides, rodenticides and nematicides are examples of classes of pesticides used to kill pests such as weeds, insects, fungi, rodents and nematodes, respectively. Herbicides can be classified as soil or plant foliage compounds. The first are absorbed by the roots, whereas the formers are absorbed by leaf tissues. Contact herbicides kill only the portion of green tissue that is contacted with the pesticide whereas systemic herbicides are translocated in a plant´s vascular system from the point of absorption to the sites of action. Systemic herbicides can be selective without affecting the growth of other plants. Herbicide's mode of action can be through photosynthesis inhibition, hormonal regulation of plant growth or through lipid and amino acids synthesis inhibition [1-3]. They can be applied at different crop stages, such as pre-sowing and pre- or post-emergence. Insecticides are usually classified as external or internal poisons (contact vs. stomach) and as fumigants if their action is through the insect's respiratory system. Insecticides can interfere with the nervous system. As such they present few problems of phytotoxicity, but of all pesticides they present the greatest acute risk to the health of human beings and fauna in the environment. Fungicides may act in a protective or

systematic manner. This type of pesticides can interfere with some enzymatic processes and can act as cell divisors and ergosterol inhibitors. Protective fungicides do not usually enter in the plants and reapplication may be required. Consequently these compounds may present higher risk of environmental contamination than the systemic fungicides.

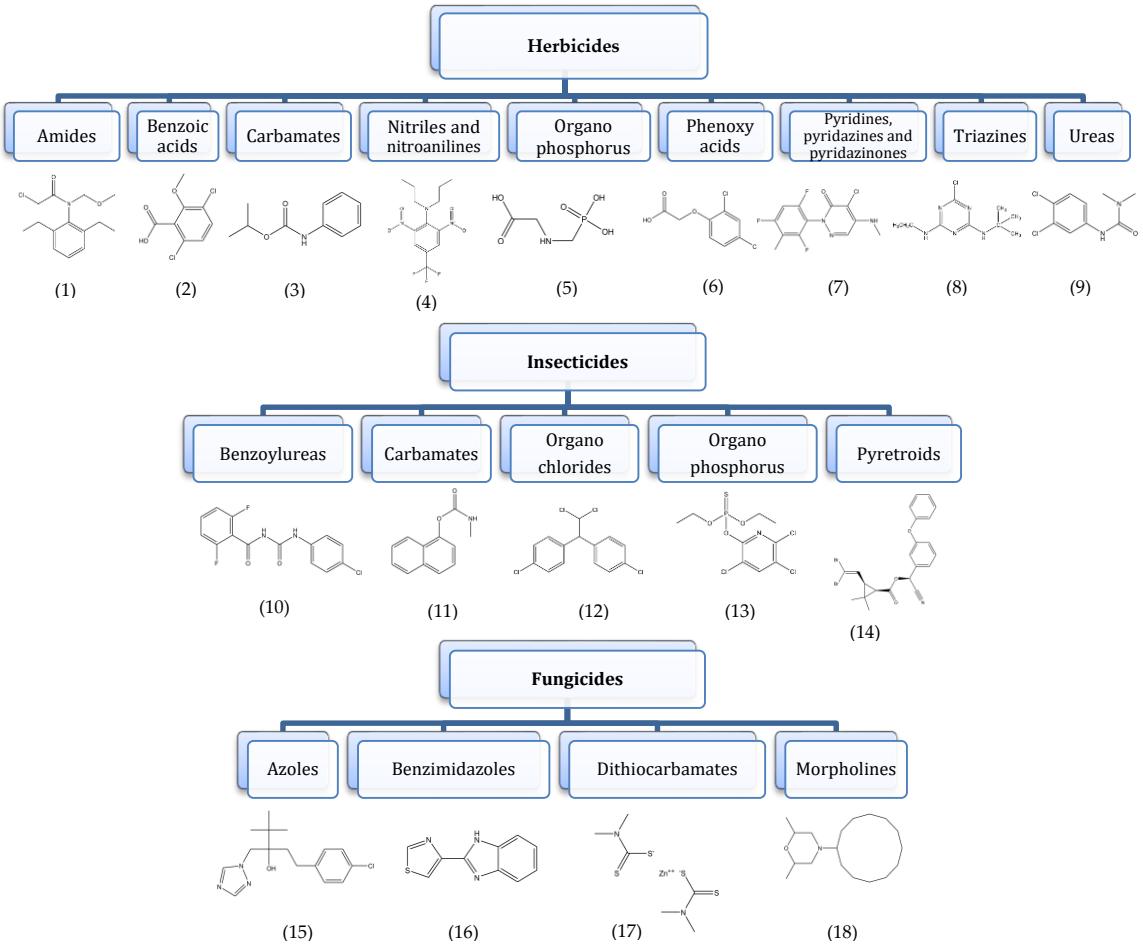

Fig. 1.1 – Classification of pesticides: pest control and chemical nature: (1) – Alachlor, (2) – Dicamba, (3) – Propham, (4) – Trifluralin, (5) – Glyphosate; (6) – 2,4-D, (7) – Norflurazon, (8) – Terbuthylazine, (9) – Diuron, (10) – Diflubenzuron, (11) – Carbaryl, (12) – *p,p'*-DDT, (13) – Chlorpyrifos, (14) – Deltamethrin, (15) – Tebuconazole, (16) – Thiabendazole, (17) – Ziram, (18) – Dodemorph.

In chemical terms pesticides are divided in two main groups: the inorganic and the organic compounds. The inorganic are those that do not contain carbon and are the least applied. Sulfur and copper sulfate are some examples. The organic pesticides are the ones containing carbon, hydrogen,

oxygen, nitrogen, phosphorous, sulfur and other elements and are subdivided into groups or classes according to their functional group. Amides, azoles, benzimidazoles, benzoic acids, benzylureas, carbamates, dithiocarbamates (organosulfur), morpholines nitriles, nitroanilines, organochlorides, organophosphorous, phenoxy acids, pyridines, and quaternary ammonium compounds, pyridazines and pyridazinones, pyrethroids, triazines, phenyureas and sulfonylureas are some examples of the complex world of the pesticides (Fig. 1.1) [1, 7]. The amount of substances with pesticidal properties is wide and its number is increasing. Pesticides classification in terms of groups offers some simplification through inter and intra-comparison and initial extrapolation of their properties and behavior.

1.1.2 Consumption indicators

In the last decades Europe has been the main consumer of pesticides followed by North & Central America, Asia, South America, Australia and Africa [8, 9]. In the European Union (EU), Belgium sells the largest quantities of pesticides per hectare (ha) of utilized agricultural area (UAA), followed by Italy, the Netherlands and Portugal [8, 10, 11]. Norway, Estonia and Sweden sold the lowest amounts of pesticides per ha of UAA [8, 10, 11]. In the EU the fungicides, herbicides and insecticides account for almost 95% of the total of pesticides used in crop production [11].

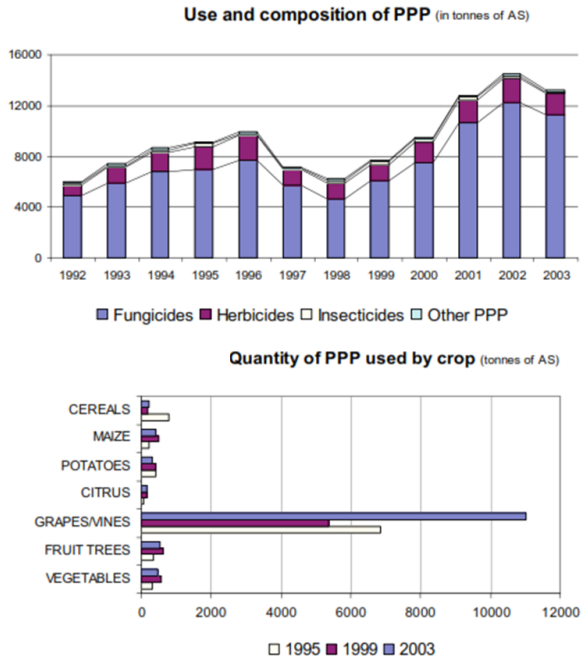

Fig. 1.2 – Pesticides consumption in Portugal: (**a**) Use and composition of pesticides in tonnes of active substance (AS); (**b**) Quantity of pesticides used by crop in tonnes of AS. Adapted from Eurostat reports [10, 11].

In Portugal from 1992 to 2003 the use of fungicides almost doubled with a temporary decrease from 1996 to 1998 (Fig. 1.2a). A high consumption of fungicides in Portugal can be explained by the predominance of grape production. As shown in Fig. 1.2b, most of total of pesticides applied to crops are used on vineyards. In terms of chemical classes, the inorganic sulfur used as fungicide in vines and vegetables dominates the Portuguese pesticide's consumption followed by the dithiocarbamates fungicides and the organophosphorus herbicides [10]. Organophosphorus insecticides are also used mainly in potato crops [10].

1.1.3 Legislation and the Water Framework Directive

Over the last 20 years regulation of pesticides has become more complex and stringent. In the European Union (EU) pesticides industry is regarded as one of the most regulated of all with a large number of directives, codes and protocols administered and advised by a vast number of committees and other bodies [9]. Pesticide legislation covers not only the conditions

concerning the placing of pesticides on the market but also the levels of pesticide residues allowed in water and food, pesticide statistics, as well as the action to be taken to promote sustainable use of pesticides and to minimize the negative impacts on human health and on environment [4, 12-19]. Until 1991, the Member States of the EU operated individually regarding registration and use of pesticides [9]. The introduction of Directive 91/414/EEC [4] aimed to coordinate the regulation of pesticides thought the EU. Directive 91/414/EEC [4] states that a plant protection product, where pesticides are included, must be tested and officially authorized. The pesticides cannot be placed on the market unless its active substance or substances are listed in Annex I of Council Directive 91/414/EEC. In foods, the concentration of pesticide residues is controlled by Regulation (EC) nº396/2005 [13]. In water, Directive 2000/60/EC [12] establishes a framework for Community action in the field of water policy.

1.1.3.1 Priority substances – The chemical status of water bodies

Directive 2000/60/EC, also known as Water Framework Directive (WFD), aims to protect the aquatic environment from pollution by preserving and improving the quality of inland surface waters, transitional waters, coastal waters and groundwater. Under WFD, Member States must adopt specific measures against pollution of surface water and groundwater to attain for good surface water status and good groundwater status. 'Good surface water status' means the status achieved by a surface water body when both its ecological status and its chemical status are at least good [12]. 'Good surface water chemical status' is defined by the WFD as the chemical status achieved by a body of surface water in which concentrations of certain compounds do not exceed the environmental quality standards (EQS) established for a list of substances, defined as priority substances (PS) and listed under Annex X of the WFD [12, 20]. With this control, WFD aims at enhancement, protection and improvement of the aquatic environment through specific measures for the progressive reduction of discharges,

emissions and losses of PS and the cessation or phasing-out of discharges, emissions and losses of the priority hazardous substances (PHS), with the ultimate aim of achieving concentrations in the marine environment approaching background values for naturally occurring substances and close to zero for man-made substances [12]. The list of priority substances to be under control was initially integrated in the WFD by Decision nº 2455/2001/EC [20]. From the total of 33 priority substances of the list, 11 were classified as ''priority hazardous substances'' [20]. The list also includes 8 other substances that were already regulated at Union level [21]. The EQSs for those substances were establish by Directive 2008/105/EC [21]. In 2013, Directive 2013/39/EU [22] amended the WFD and Directive 2008/105/EC [21] as regards priority substances in the field of water policy. New substances were included in the list of priority substances making a total of 45 substances or group of substances that will be under strictly surveillance in the next decade. Among those substances, 27 compounds have been used as pesticides.

1.1.3.2 Sediments and biota

An important aspect of the Directive on EQSs is the choice of the matrix for the monitoring of the priority substances [22]. The established EQSs were set for surface waters, however, if justified, EQSs can be established at a national level in sediments and biota [21-23]. Belgium, Spain (Distrito Fluvial da Cataluña), Italy and Norway are a good example where sediments EQSs were established [24, 25]. The introduction of sediments and biota as analytical matrix has the objective to assess the long-term impacts of the possible anthropogenic activities and also to ensure that the existing levels of contamination do not increase to a stage that pose a threat to the environmental and human health [23]. In marine and lentic water bodies, sediments are the recommended matrix for the assessment of the chemical level of some metals and some hydrophobic compounds. In dynamic lotic water bodies suspended particulate matter (SPM) is the recommended matrix

because of the high variability of the water bodies [23]. Sediments are also the recommended matrix for trend monitoring. In this type of environmental compartment the changes in pollution are not as fast as in the water column and reliable long-term comparisons can be carried out [23, 26]. The selection of the organic priority substances to be monitored in sediments or SPME is based on the octanol-water partition coefficient (k_{ow}). Substances with a $\log k_{ow} > 5$ should preferably be measured in sediments, or in SPM, while compounds with a $\log k_{ow} < 3$ should preferably be measured in water [23]. For compounds with a $\log k_{ow}$ between 3 and 5, the sediment matrix and SPM is optional and will depend on the level of contamination. If the level of contamination for a hydrophobic compound is unknown or expected to be low, sediment should be an additional monitoring compartment due to possible accumulation effects [23]. The monitoring of organic compounds should be performed when the biomagnification factor (BMF) is >1 or when bioconcentration factor (BCF) is >100. If no valid measured BMF and BCF is available, $\log k_{ow} > 3$ can be considered as an indicator for bioaccumulation potential. For the PPs such as hexachlorobenzene, hexachlorobutadiene, dicofol and heptachlor and its metabolite heptachlor epoxide, the EQSs in biota were established by the latest Directive 2013/39/EU [22]. Their concentration in fish must not exceed 10, 55, 33 and 0.0067 $\mu g kg^{-1}$ (wet weight), respectively [22].

1.1.4 The priority pesticides and their key physicochemical properties

The pesticides listed under Annex X of the WFD were classified as priority substances because they are toxic, persistent and liable to bioaccumulate. The list of such pesticides, herein defined as ''priority pesticides'' (PPs), is presented in Table A1 together with some relevant physicochemical properties that are used to predict/evaluate their environmental fate and distribution. Under the WFD the PPs were classified as PS or PHS and grouped as shown in Table A.1 (Annex A). The pesticides that have been classified as Persistent Organic Pollutants (POPs) under

Stockholm Convention are also included in Table A.1 [27, 28]. Most of POPs are listed as priority substances except chlordane, chlordecone, mirex, quintozene and toxaphene. POPs like aldrin, dieldrin, endrin, isodrin and *p,p'*-DDT do not have the designation of priority substances. Those pesticides were early regulated by other directives at the Union Level and further included into the group of the 8 other substances under Annex X of the WFD [12, 21]. Most of the PPs have been banned, however, due to their persistence and extensive use in the past their presence in the environment is still a reality.

Different classes of pesticides with different applications are found in the list of the PPs (Annex A, Table A.1). Organochloride compounds with an insecticide action are the predominant class. Such class of pesticides was very popular in the past due to their efficacy on pest control. Unfortunately, they became more popular due to their toxicity to humans and to other non-target organisms [29, 30]. Among the PPs (Annex A, Table A.1), endrin is the most toxic and can be classified as super toxic with an acute oral LD_{50} (rats) lower than 5 $mgkg^{-1}$ [29]. Endosulfan, aldrin, dieldrin, isodrin, heptachlor, toxaphene and the organophosphorous chlorfenvinphos and dichlorvos are classified as extremely toxic with an acute oral LD_{50} between 5 - 50 $mgkg^{-1}$ (Table A1) [29, 31]. The others PPs, are classified as very toxic, moderately toxic or slight toxic with values of LD_{50} between 50 - 500 $mgkg^{-1}$, 500 - 5000 $mgkg^{-1}$ and 5000 - 15000 $mgkg^{-1}$, respectively (Annex A, Table A.1) [29, 31].

The stability of pesticides in terms of resistance to photolysis, hydrolysis and microbial degradation is to a large extent a function of their chemical structure. The variation in ring structures and the different types of chemical bonds largely determine their stability. Of particular note is the stability of chlorinated ring structures of the organochlorine pesticides resulting in a high persistence in the environment [9, 31]. Resistance to metabolic breakdown in the tissues of non-target species has also allowed these compounds to accumulate in food chains [9, 31, 32]. The accumulation of the PPs is

intrinsically related with its lower solubility in water and high tendency to be sorbed by organic matter. As shown in Table A.1, the majority of the PPs are insoluble or slightly soluble in water, except dichlorvos. The octanol-water partition coefficient (k_{ow}) is also an important parameter used to predict compounds hydrophobicity [9]. $Logk_{ow}$ is applied as a guideline to select the substances that shall be monitored in sediments and/or biota under the WFD [33, 34]. Most of the PPs have a log k_{ow}> 3 (Table A1). Such compounds have the tendency to be sorbed by the organic matter present in soils and sediments. Atrazine, diuron, isoproturon, simazine, and dichlorvos are an exception. Their tendency to accumulate in sediments and biota is lower and water will be the best matrix for environmental fate studies and water quality characterization [23]. The vapor pressure is also another important physicochemical property. Compounds with a high vapor pressure are generally volatile and may readily enter the atmosphere once applied in the field [35]. Hexachlorobutadiene, heptachlor, quintozene, trifularin, the cyclodiene organochlorides and chlorpyrifos are good examples [34-37]. They can be easily transported through air after application. Such compounds are insoluble or slightly soluble in water and will be sorbed by the organic matter present in soils ($logk_{ow}$> 3). The compounds can be taken from soil by plants roots but they will not be translocated to the leaves due to their high hydrophobicity. Consequently the compounds can exhibit significant vapor transport if applied to the soil surface under warm conditions and moist surface soil. Combination of low water solubility and high vapor pressures yields high values of the Henry's Law constant (H). If release to water, pesticides with high values of H at a certain temperature will have the tendency to volatilize. However, volatilization can be reduced by adsorption to sediments and bioorganic matter present in aquatic systems. The actual losses of the compounds in the field can be highly variable and are still difficult to evaluate. Nonetheless, their physicochemical properties are an essential first indication of the likely dissipation of the compounds in the

environment. They are also a valuable tool for the development of analytical methods.

1.2 Coastal lagoons: a potential sink of pesticides

A coastal lagoon is a shallow water body separated from the ocean by a barrier, connected at least intermittently to the ocean by one or more restricted inlets [38]. Lagoons constitute 13% of coastal regions globally, range in area from <0.01 km^2 to >10,000 km^2, and are typically <5 m deep [38]. They are formed and maintained through sediment transport processes. Coastal lagoons are highly productive ecosystems. They contribute to the overall productivity of coastal waters by supporting a variety of habitats, including salt marshes, sea grasses and mangroves [38]. The continental inputs in the coastal lagoons are mainly characterized by river waters and, sometime, by ground water or rain water that drain the surrounding soils. These waters not only carry large amounts of particulate material in the form of clay particles and organic detritus but also dissolved material in the form of dissolved organic matter (DOM) and nutrients arising from human activity in the vicinity of the lagoons (fertilizers, pesticides, domestic and industrial effluents, etc.) [39]. Water quality and its volume in lagoons is influenced by the rate at which the lagoon loses or gains water from the evaporation, precipitation, groundwater input, surface runoff, and exchange with the ocean [38]. In general, because of the restricted exchange with the ocean, coastal lagoons trap inorganic sediments and organic matter from distinctive sources, serving as a sink of many and different organic contaminants like the pesticides. Coastal lagoons fall under the WFD which objective is to achieve 'good surface water chemical status' in EU. The monitoring of pesticides with a classification of priority substances is therefore a requirement. Moreover, there is a scientific agreement that climate change will have a pervasive influence on the future demand, supply and quality of fresh water resources in the Mediterranean, and will add pressure to water, environment resources

and coastal systems [40]. Because of their shallow waters and low volume compared to the adjacent sea, coastal lagoons will probably be more affected by global changes and external drivers such as temperature, precipitation or UVB radiations changes. Understanding the fate and the distribution of the PPs in coastal lagoons would help in the planning of the monitoring programs, on the actions to be taken under climate changes and consequently on the protection of such sensible aquatic ecosystems against pollution.

1.2.1 Óbidos Lagoon

1.2.1.1 An environmental and socio-economical overview

In Portugal, Óbidos Lagoon (OL) is a shallow coastal lagoon located on the Western coast (Fig. 1.3a) with a mean depth of 1 m and a wet area of 7 km^2 [41]. It is permanently connected to Atlantic Ocean through a narrow inlet. The lagoon is formed by a central body with two elongated bays: the *Barrosa* branch oriented SE with depths not exceeding 1.5 m and the other one, the *Bom Sucesso* branch at SW with a depth of about 4.5 m [41-43]. Areas with different morphological and sedimentary characteristics were identified in the lagoon: several sand banks, narrow channels and strong currents in the lower and middle lagoon; and muddy bottom sediments in the inner branches *Barrosa* and *Bom Sucesso* [42, 43]. OL supports a variety of habitats, and several aquatic and migratory birds species can be found in the lagoon [44]. The lagoon system comprises the reed beds, the salt marsh and the lagoon itself [45, 46]. There is a predominance of areas occupied by eucalyptus, on the slopes around the lagoon and upstream of the watershed can be defined three preferred occupation groups: the first, between *Óbidos* and *Bombarral*, it has preferred occupation orchard; the second correspond to intermediate quotas, extending from *Bombarral* to the *Cadaval* and has predominance of the vineyard; the third, in the southern part of the basin of *Cadaval* county the eucalyptus is the dominant plantation [45, 46]. OL serves as a base level for a hydrographic net, with about 430 km^2, that extends

through the municipalities of *Cadaval* and *Bombarral* (Fig. 1.3b) [47, 48]. The hydrographic basin can be subdivided in several sub-basins, the most important being those of Real and Arnóia rivers (Fig. 1.4) [49]. The Arnóia River with its confluent the Real River, is discharged in the middle of the two branches and contributes with about 90% of the freshwater that enters into the lagoon [50, 51]. Other small streams enter in the lagoon: the Cal River at the *Barrosa* branch and the stream Vala do Ameal at the *Bom Sucesso* branch (Fig. 1.4). In spite of the discharge of several streams, freshwater inputs in OL is considered low with annual average flow of 3 m^3s^{-1} [42, 43]. Arnóia River is the main source of sediments and has created and extensive sand bank in the center of the upper lagoon [50].

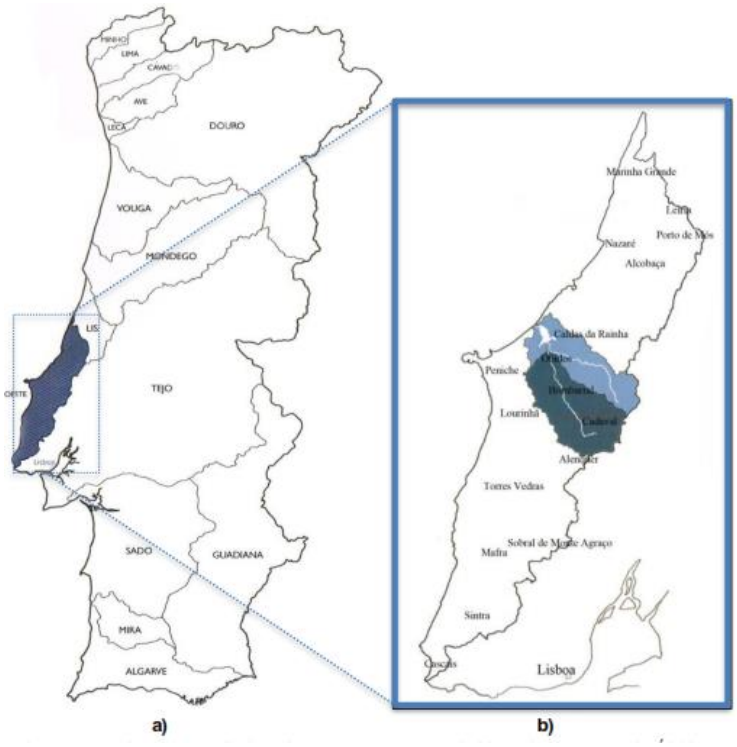

Fig. 1.3 – Map of (**a**) Portugal with emphasis of the Western coast where Óbidos Lagoon is located and (**b**) the hydrographic basin of Óbidos Lagoon. Adapted from references [41, 47-49].

Fig. 1.4 – Map of the sub-basins including Óbidos Lagoon and Arnóia River (Bacia do Rio Arnóia). Water quality: blue - Excellent; green – Good; yellow - Reasonable; orange - Mediocre; red – Bad; dark gray – Undefined. Adapted from the ARH Tejo report [49].

OL is a Western European coastal lagoon and is of considerable ecological and economical interest [51]. The urban centers near OL are *Óbidos* and *Caldas da Rainha* with approximately 12,000 and 52,000 habitants, respectively. OL has a significant extension being used for the practice of several sports like sailing and windsurfs [45]. The main economic activities are shellfish, agriculture, livestock and tourism [45, 49].

1.2.1.2 Identification of environmental concerns

The Óbidos Lagoon, as any coastal lagoon, is potentially exposed to anthropogenic activites acting as a sink for pesticides, heavy metals and nutrients. The anthropogenic impacts in OL are mainly due to agricultural and industrial (livestock and wineries) pressures [49]. Pesticides, or any other synthetic contaminant may enter the lagoon by discharges of polluted streams and/or by contaminated soils through runoff. Until 2006, the urban and industrial wastewaters from *Caldas da Rainha* were discharged in Cal River that enters into the lagoon through *Barrosa* branch. Nowadays, the wastewater effluents are released directly in the coastal zone adjacent to the

lagoon through a submersed outfall [43]. The impact of the urban and industrial wastewaters on the lagoon might have decreased. However, the latest diagnostic of the Arnóia sub-river basin shows that 75% of the water bodies are below the reference value of ''good water status´´ as illustrated in Fig. 1.4 [49]. In fact, OL was classified as a sensitive zone relatively to nutrients with eutrophication problems mostly in the *Barrosa* branch [43, 49]. Those problems are potentially substantiated by the difficulties of water renewing, particularly in the branches, leading to a high accumulation of nutrients [41]. The nutrient loading and the longer residence time of water in inner branches (24 – 26 and 4 – 10 days in *Bom Sucesso* and *Barrosa*, respectively) in comparison to the middle/lower lagoon (1 – 4 days) favors the macroalgal (*Ulva* sp. and *Enteromorpha* sp.) cover as well as the accumulation of organic matter in sediments [43]. Although no priority substances were detected in the water bodies of the Arnóia sub-river basin, a study revealed the presence of the PPs dieldrin and *p,p'*-DDT in sediments of the inner part of OL [41]. Possible source of PPs in the lagoon includes the agricultural surrounding area and the livestock. PPs are highly hydrophobic and their tendency to be sorbed by organic matter is high. The presence of compounds like the PPs in sediments can lead to problematic scenarios. Sediments are the habitat of benthic biota. PPs are toxic and are a threat to biota and global populations. Exposure to contaminated sediments can result in a decrease of survival, reduced growth and impaired reproduction of many aquatic organisms [52]. Contamination has also a negative impact on sediment management. Handling of contaminated material, e.g. in the cases of dredging, is much more expensive than handling clean material [53]. Moreover, re-suspension of the contaminated sediments caused by storms and dredging activities can increase pesticide's concentration on the water column and consequently their fate, transport, availability and uptake by other aquatic and non-aquatic organisms.

1.3 Analytical tools

Environmental Quality Standards (EQSs) are the references values used to evaluate the chemical status of the water bodies. The EQS Directive [22] establishes the maximum acceptable concentration and/or annual average concentration for the priority substances, which if met, allows the chemical status of the water body to be described as 'good'. Rapid, sensitive and rigorous analytical methods are thus a valuable tool for water monitoring and subsequent status classification. Although a great number of official methods have been published, especially for water analysis, the studies have shown that for some pesticides their performance does not comply with the technical quality standards [54, 55]. The threshold limits set by EQSs for waters are so low for some of the priority substances that, by default, this implies high quality of analytical data [54]. Gas chromatography (GC) using selective electron capture detector (ECD) and mass spectrometry detector (MSD) have been the most applied technique for the analysis of less polar PPs while liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been applied to more polar and thermo-labile pesticides [1, 56]. In terms of sample preparation the main options have been soxhlet extraction followed by sample clean-up and pre-concentration using florisil, alumina and/or silica chromatographic columns and also the pre-packed solid-phase extraction (SPE) cartridge [56-62]. Other extraction techniques like ultrasound-assisted extraction (UAE), stir bar sorptive extraction (SBSE), pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and liquid-phase microextraction (LPME) are a green and growing alternative [61-66]. To fulfill the validation requirements, the developed analytical methods must meet the technical specifications for chemical analysis and monitoring of water status, sediment and biota stated in Directive 2009/90/EC [67]. The use of standardized methods is recommended in water monitoring programs, however, only a few standard methods exist for sediment analysis and none

of them is for pesticides screening [23, 68]. To overcome this gap, existing soil reference methods may be applied as long as validation on the appropriate matrix is carried out [23, 69]. Only two methods are available for soil pesticides analysis and together they do not include the entire listed PPs [69]. Moreover, sediment and soil certified reference materials (CRM) are not available for all listed PPs. The validation of the analytical methods must be carried out by proficiency tests, which increases the time and the costs of the entire validation process. The development of analytical methods for the simultaneous determination of entire spectrum of PPs in sediments that meet the technical specification is still a gap that must be fulfilled.

1.4 Motivation and thesis outline

1.4.1 Motivation

The leitmotiv for this project was the knowledge gap concerning environmental pesticides fate and their impact on the aquatic organisms as well as the lack of sensitive analytical methods for their measurement. As semi-closed systems, the coastal lagoons can act as a sink for pesticides. In Portugal, the Óbidos Lagoon receives drainage waters and pesticides from agriculture fields and agro-industrial activities being for those reasons the selected area. A better understanding of pesticides distribution in this area will be a good advance in the extrapolation to other problematic areas. As coastal waters, Óbidos Lagoon falls under the Water Framework Directive. The monitoring of priority pesticides in river basins is therefore a demand. An understanding of pesticides distribution and effects can provide useful information to the support of environmental quality measures. Value judgments about ecosystems pollution require a quantitative assessment of the different matrices. In this context, the objectives of this study were:

- to review and update the information regarding the analysis of priority pesticides (PPs) in water and the performance of the analytical methods;

- to develop environmental friendly analytical methods for the determination of the different classes of PPs in sediments and biota, especially the macroalgae *Ulva* sp.;
- to evaluate the occurrence and retention of the PPs in sediments and in *Ulva* sp. in Óbidos Lagoon;
- to investigate the effect of dissolved organic matter and the salinity on the fate of the PPs;
- to estimate the impact under climate changes on the mobility and fate of PPs.

1.4.2 Thesis structure

This thesis is organized in eight chapters, including the current **introductory chapter (Chapter 1)**, which was a general introduction about pesticides, their classification, global consumption and physicochemical properties as well as the European legislation applied to pesticides with special emphasis to pesticides classified as priority substances under the WFD. Chapter 1 also describes the importance of coastal lagoons and the associated environmental concerns in particular for Óbidos Lagoon and also the importance of the analytical tools for the management and protection of coastal lagoons against pesticides pollution. The motivation of the presented work was also described as well as thesis's layout and outputs.

Chapter 2 updates the information concerning the monitoring the PPs and the technical specifications required for their measurement in water bodies. Chapter 2 also reviews the performance of the new developed liquid-phase microextraction techniques and compares its performance with the analytical specifications established for PPs water monitoring under the WFD.

Chapter 3 and **chapter 4** report the development, optimization and validation of two analytical methods for the determination of the PPs in sediments and *Ulva* sp., respectively. Both methods are a valid and a green alternative to the traditional analytical methods.

Chapter 5 evaluates the occurrence and the pathways of the PPs in sediments of Óbidos Lagoon.

Chapter 6 evaluates the influence of the dissolved organic matter (DOM) on the processes of transformation/dissipation of the insecticide chlorpyrifos classified as a PPs. Findings of chlorpyrifos photodegradation and volatilization kinetics in saline waters are reported.

Chapter 7 describes the development of an analytical method for the selective fractionation of DOM. Different fractions were obtained by elution of DOM from natural waters using different mixtures of polar solvents. The technique was applied to the comparative characterization of saline and wetland waters.

Chapter 8 reports a general discussion of the results and presents some guidelines for future research works.

1.4.3 Thesis output

1.4.3.1 Papers published in International Scientific Journals

- M. I. Pinto, G. Sontag, C. Vale, J. P. Noronha, Pathways of priority pesticides in sediments of coastal lagoons: The case study of Óbidos Lagoon, **Mar. Pollut. Bull.**, submitted.
- M. I. Pinto, Hugh D. Burrows, C. Vale, G. Sontag, J. P. Noronha, William J. Cooper, Barbara A. Cottrell, A New 2D separation for the characterization of dissolved organic matter, **Geochim. Cosmochim. Acta**, to be submitted.
- M. I. Pinto, R. Salgado, Barbara A. Cottrell, William J. Cooper, Hugh D. Burrows, C. Vale, G. Sontag, J. P. Noronha, Influence of dissolved organic matter on the photodegradation and volatilization kinetics of chlorpyrifos in coastal waters, **J. Photoch. Photobio. A** 310 (2015), 189 – 196.
- M. I. Pinto, C. Micaelo, C. Vale, G. Sontag, J. P. Noronha, Screening of priority pesticides in *Ulva* sp. seaweeds by selective pressurized solvent

extraction before gas chromatography with electron capture detector analysis, **Arch. Environ. Cont. Tox.** 67 (2014), 547 – 556.

- M. I. Pinto, G. Sontag, C. Vale, J.P. Noronha, Effects of ultrasonic irradiation and direct heating on extraction of priority pesticides from marine sediments, **Int. J. Environ. Anal. Chem.** 93 (2013), 1638 – 1659.
- M. I. Pinto, Gerhard Sontag, R.J. Bernardino, J.P. Noronha, Pesticides in water and the performance of the liquid-phase microextractions based techniques. A review, **Microchem. J.** 96 (2010), 225 – 237.

1.4.3.2 Participation in National and International Conferences

- M. I. Pinto, H. D. Burrows, C. Vale, G. Sontag, J. P. Noronha, W. J. Cooper, B. A. Cottrell, A new 2D separation for characterizing dissolved organic matter, *NOM 6 - IWA Specialist Conference on Natural Organic Matter in Drinking Water,* 7 – 10th September 2015, Malmö, Sweden (oral communication).
- M. I. Pinto, R. Melo, H.D., Burrows, W. J. Cooper, G. Sontag, C. Vale, J. P. Noronha, Degradation of tetracyclines by gamma irradiation advanced oxidation processes (AOPs): Influence of the different radical species and the absorbed dose, *13th IUPAC International Congress of Pesticides Chemistry*, August 10 – 14t, 2014, San Francisco, USA.
- M. I. Pinto, C. Micaelo, G. Sontag, C. Vale, J. P. Noronha, Macroalgas como bioindicadores de poluição por pesticidas, *12º IUPAC Congresso da Água/16º Encontro de Engenharia Sanitária e Ambiental (ENASB)/ XVI Simpósio Luso-Brasileiro de Engenharia Sanitária e Ambiental (SILUBESA)*, 5 a 8 Março 2014, Lisboa, Portugal.
- M. I. Pinto, C. Micaelo, A. Ferreira, C. Vale, G. Sontag, J.P. Noronha, The screening of priority pesticides in green seaweeds from Óbidos Lagoon, *7th European Conference on Pesticides and Related Micropollutants in the*

Environment and 13th Symposium on Chemistry and Fate of Modern Pesticides, 7 – 10th of October, 2012, Porto, Portugal.

- Pesticides in Water, Sediments and Biota of Óbidos Lagoon, Portugal, M. I. Pinto, A. Ferreira, C. Vale, G. Sontag, J.P. Noronha, *36th International Symposium on High-Performance and Liquid Phase Separations and Related Techniques*, 19 – 23rd June, 2011, Budapest, Hungary.

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2

Pesticides in Water and the Performance of the Liquid-Phase Microextractions based Techniques. A Review

Highlights

- Review of the recently innovations of liquid-phase microextraction (LPME) techniques and their performance.
- Update of information regarding monitoring of priority pesticides and the technical specifications for their measurement in water.

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Abstract

The control of pesticides in surface, drinking and groundwater is nowadays a real necessity. In the European Community, their concentration must comply with the established parametric and environmental quality standards (EQSs). Regarding the new legislation, this article updates the information concerning the monitoring of pesticides and the technical specifications for their measurement in water samples where ultra-sensitive analytical methods are required. For some compounds, like pesticides, there is still a need to improve the performance of the existing methods. High sensitive techniques like gas chromatography tandem mass spectrometry (GC-MS/MS) and liquid chromatography coupled with mass spectrometry (LC-MS) have been developed. However, for most of the substances present at trace and ultra trace-levels the extraction and preconcentration steps are so far essential for their detection. Advances at a micro scale have been made and different types of microextractions are being developed. Liquid-phase microextraction (LPME) is an example. The study of this technique has increased in the last years and some innovations have been recently reported for pesticides water analysis. This article reviews the new developed LPME-based techniques and compares its performance with the analytical specifications established for pesticides water monitoring. The results show that LPME-based techniques can be a promising tool to improve the nowadays performance of methods used in pesticides water control.

Keywords: Pesticides; Water monitoring; Environmental Quality Standards; Methods performance criteria; Chromatography; Liquid-phase microextraction.

2.1. Introduction

It is well known that pesticides have the potential to prevent and control harmful organisms being a powerful tool to agricultural problems. It has been estimated that around one-third of the crop production would be lost if chemical substances were not applied against pests [1]. Pesticides have also been used in non agricultural sectors such as wood preservation, disinfection or household uses. In spite of the several advantages, some pesticides can be toxic to humans and animals and their continuous application is causing serious problems of environmental and food contamination. The legislation in this area covers not only the conditions concerning the placing of pesticides on the market but also the levels of pesticide residues allowed in waters and food as well as the actions needed to promote sustainable use of pesticides [2-7]. In the European Community (EC), Directive 2000/60/EC [2] establishes a framework for the protection of waters. This Water Framework Directive (WFD) aims to reduce and to eliminate the presence of substances in the aquatic environment that have been considered by the Commission as toxic, persistent and liable to bio-accumulate. Such substances, named as priority substances and priority hazardous substances, are listed in Annex X of the WFD together with its permissible limits or environmental quality standards (EQSs) [2, 8]. Not only the pesticides that are classified as priority substances are subject of control, other pesticides that are likely to be present in drinking and groundwater have also to be analyzed. The state of art in monitoring the priority substances in waters have been reviewed [9, 10]. This article updates the information regarding the screening of pesticides in surface, drinking and groundwater. The data reviewed shows that ultra-sensitive analytical methods are mandatory since the water tolerable limits for most of the pesticides are in the levels of parts per billion and in some cases parts per trillion [8, 11-13]. To be detectable and quantified, the substances present at trace levels must be extracted and concentrated. Although environmental unfriendly, liquid-liquid extraction (LLE) is a classic preconcentration

technique that has long been used in some standard analytical methods developed for routine analysis of pesticides [1, 9, 14-23]. Good recoveries and precision have been achieved with such methods. However, as pointed out by P. Lepom et al. [9] the existing standards for the analysis of some priority substances, where some pesticides are included, are not sensitive enough to conduct the actual compliance monitoring. The application of high sensitive analytical systems like gas chromatography tandem mass spectrometry (GC-MS/MS) and liquid chromatography coupled with mass spectrometry (LC-MS) has been a powerful option, nevertheless, a preconcentration of the compounds present mostly at trace levels prior to analysis is absolutely necessary. High recoveries and sample enrichments of the extracted substances have been obtained with new solvent microextraction techniques. Liquid-phase microextraction (LPME), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) are examples. LPME is simple, rapid and inexpensive when compared with other extraction techniques. In addition, it can be applied to a wide range of compounds including pesticides. It is highly compatible with chromatographic methods and improvements have been made after its introduction in the mid-to-late 1990s [24-26]. LPME techniques seem to be a promising tool for the development of very sensitive standard procedures since the associated limits of detection (LOD) of the chromatographic methods can be at the levels of nanograms per liter (ngL^{-1}). Thus, the aim of this study is to review the recently innovations of liquid-phase microextraction (LPME) techniques applied to chromatographic water pesticides analysis and to compare its results with the performance method criteria established for water monitoring status [2, 11-13]. Emphasis will be given to the LOD of pesticides classified as priority substances.

2.2. Control of pesticides and the performance criteria for their measurement

The presence of pesticide residues in the environment it is a reality and the control and monitoring of contaminants a matter of health protection. In the last decades there has been an enormous effort by companies and authorities to study and collect all possible information concerning the safe introduction of pesticides in the market and their release to the environment [1, 27-29]. This sort of information has been valuable for monitoring water policy purposes [2, 8, 29, 30]. Under the WFD, specific measures must be adopted against pollution of surface water and groundwater to attain ''good water status'' by the end of 2015. To reach this main objective, ecological and chemical parameters must be met and pollution sources controlled [2, 11, 31-45]. The presence of some substances classified as priority substances must be progressively reduced while for some other substances, which fall under the definition of priority hazardous substances, the occurrence in the aquatic environment must be eliminated. From the total of 33 priority substances listed by WFD, 11 were classified as priority hazardous substances [8]. Table 2.1 summarizes the pesticides that must be controlled in surface waters together with their EQSs. Some of them were not listed as priority substances however, their monitoring was maintained since they fall under the scope of other Directives [8]. For health reasons, drinking and groundwater are also subject of control [2, 11, 12]. The list of individual pesticides to be monitored in those types of water is published every year by the competent authorities of each Member State. Only those pesticides which are likely to be present in a given water supply need to be supervised. For groundwater, and while a revision of the Drinking Water Directive is being prepared, the limits set for both type of waters are 0.5 μgL^{-1} for the sum of all pesticides and 0.1 μgL^{-1} for each compound [11, 12, 46, 47]. Aldrin, dieldrin, heptachlor and the metabolite heptachlor epoxide are an exception. The limit is 0.03 μgL^{-1} [11]. These substances are highly toxic to humans. For aldrin and dieldrin the

established EQS are even lower than the health safe limit. Heptachlor is not among the list of priority substances but is listed under the Stockholm Convention on Persistent Organic Pollutants (POPs), among other pesticides that are being restricted and will be banned in the next years [48-50]. To meet all the requirements of a good water chemical status, communities must have means of measure the undesirable substances in water samples. Rapid, sensitive and rigorous analytical methods are thus a valuable tool for quality assurance of aquatic ecosystems. To be comparable, the results must be precise, accurate and in agreement with official technical specifications for chemical analysis and monitoring of water status [13]. Even though the methods must be validated and well documented in accordance with EN/ISO 17025 or any equivalent standard, the analytical methods applied to quantify the priority substances must follow the recently established minimum performance criteria [13, 51]. For a method to be approved, the limits of quantification (LOQ) must be equal or below a value of 30% of the relevant EQS, as summarized in Table 2.1, and the uncertainty of the results must be 50% or below the estimated EQS with a coverage factor of 2 (k=2) corresponding to a level of confidence of approximately 95%. For drinking waters, the performance criterion is based on the limit of detection (LOD). This limit must be equal or lower than a value of 25% of the tolerable limit which means that for individual pesticides the LOD should be 25 ngL^{-1} [11]. In this study especially attention will be given to the LOD of methods. However, enforcements should be made at the scientific community to publish not only the LOD but also the LOQ and the uncertainty of methods since the established performance criteria for the analysis of the priority substances is essentially based on such parameters.

Table 2.1 – Environmental quality standards (EQS) for pesticides in surface waters, their mandatory LOQ and the standard methods applied (AA – annual average concentration or the arithmetic mean of the concentration measured at different times during the year for protection against long-term exposure; MAC – maximum allowable concentration at any representative monitoring point within the water body for protection against short- term exposure; n.a. – not applicable) [8-13, 49-50].

Priority Substance	CAS(1) Number	AA-EQS Inland surface water (μgL^{-1})	AA-EQS Other surface water (μgL^{-1})	MAC-EQS Inland surface water (μgL^{-1})	MAC-EQS Other surface water (μgL^{-1})	LOQ* (μgL^{-1})	Uncertainty ($\pm\mu gL^{-1}$)	POPs	Standards methods
Alachlor Chloroacetanilide Herbicide	15972-60-8	0.3	0.3	0.7	0.7	0.09	0.15	-	EN ISO 6468:1997 ISO1370:2000 EPA 8081B:2000
Atrazine Triazine Herbicide	1912-24-9	0.6	0.6	2.0	2.0	0.18	0.3	-	EN ISO 1369:1997 EN ISO 0695:2000 ISO 11370:2000 EPA 527:2005 EPA 8141A:1994
Chlorfenvinphos Organophosphorus – Insecticide	470-90-6	0.1	0.1	0.3	0.3	0.03	0.05	-	EN ISO 12918:2000 ISO 11370:2000 EPA 8270D:1998
Chlorpyrifos Organophosphorus – Insecticide	2921-88-2	0.03	0.03	0.1	0.1	0.009	0.015	-	EN ISO 12918:2000 EPA 257:2005 EPA 8141A:1994
Cyclodiene - Insecticides:		$\sum = 0.01$	$\sum = 0.005$	n.a	n.a	0.0015	0.0025		
Aldrin(2)	309-00-2							Yes	EN ISO 6468:1997 EPA 8081B:2000 EPA 8270D:1998 EPA 525.2: 1995
Dieldrin(2)	60-57-1							Yes	EN ISO 6468:1997 EPA 8081B:2000 EPA 8270D:1998

Endrin[2]	72-20-8							Yes	EN ISO 6468:1997 EPA 8081B:2000 EPA 8270D:1998 EPA 525.2: 1995
Isodrin[2]	465-73-6							-	EPA 8081B:2000 EPA 8270D:1998
DDT Total[2] Organochloride Insecticide	n.a	0.025	0.025	n.a	n.a	0.0075	0.01	Yes	EN ISO 6468:1996 EPA 8081B:2000
p,p'-DDT[2] Organochloride Insecticide	50-29-3	0.01	0.01	n.a	n.a	0.003	0.005	Yes	EN ISO 11369:1997 EPA 8325:1996 EPA 525.2: 1995
Diuron Urea Herbicide	330-54-1	0.2	0.2	1.8	1.8	0.06	0.1	-	EN ISO 11369:1997 EPA 8325:1996
Endosulfan[3] Organochloride Insecticide	115-29-7	0.005	0.0005	0.01	0.004	0.00015	0.00015	Nominated for addition	EN ISO 6468:1997 EPA 8081B:2000 EPA 525.2: 1995
Hexachlorobenzene[3] Organochloride Fungicide	118-74-1	0.01	0.01	0.05	0.05	0.03	0.005	Yes	EN ISO 6468:1997 EPA 8270:1998 EPA 8081B:2000 EPA 8270:1998
Hexachlorobutadiene[3] Fungicide	87-68-3	0.1	0.1	0.6	0.6	0.03	0.005	-	EN ISO 10301:1998 EPA 8270:1998 EPA 8021B:1996
Hexachlorocyclohexane[3] Organochloride Insecticide	608-73-1	0.02	0.002	0.04	0.04	0.0006	0.001	Yes	EN ISO 6468:1997 PA 8081B:2000 EPA 8270:1998

Isoproturon Urea Herbicide	34123-59-6	0.3	0.3	1.0	1.0	0.09	0.15	-	EN ISO 11369:1997
Pentachlorophenol Insecticide, fungicide and herbicide	87-86-5	0.4	0.4	1	1	0.12	0.2	-	EN ISO 12673:1999 ISO 8165-1:1992 ISO 8165-2:1999 EPA 8270:1998
Simazine Triazine Herbicide	122-34-9	1	1	4	4	0.3	0.50	-	EN ISO 10695:2000 ISO 11370:2000 EPA 8141A:1994
Trifluralin 2,6-dinitroanilide Herbicide	1582-09-8	0.03	0.03	n.a	n.a	0.009	0.15	Yes	EN ISO 10695:2000 ISO 11370:2000 EPA 8081B:2000 EPA 8270:1998

(1) CAS – Chemical abstract service; (2) – This substance is not a priority substance but one of other pollutants for which the EQS are identical to those laid down in the legislation that applied prior to 13 January 2009 (entry into force of Directive 2008/105/EC); (3) – priority hazardous substance.

DDT total comprises the sum of the isomers 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (CAS number 50-29-3; EU number 200-024-3); 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (CAS number 789-02-6; EU number 212-332-5); 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (CAS number 72-55-9; EU number 200-784-6); and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (CAS number 72-54-8; EU number 200-783-0).

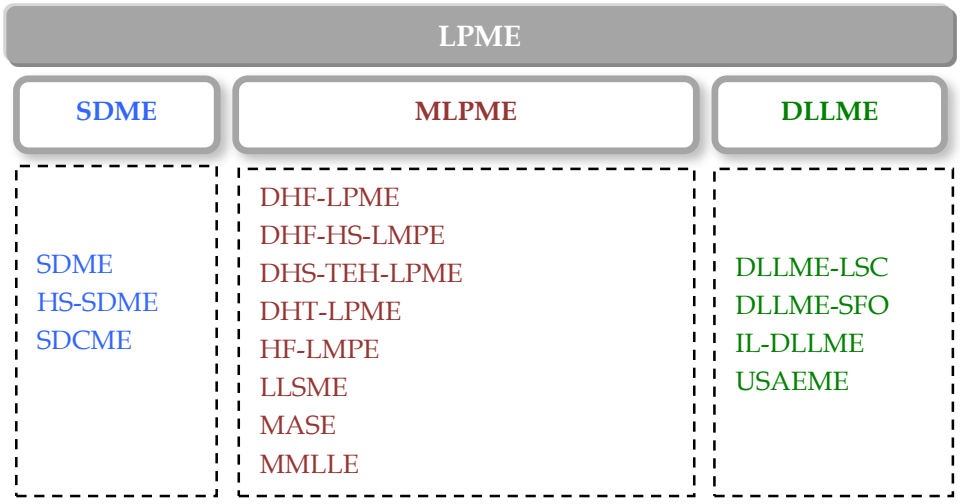
The term inland surface waters includes rivers, lakes and related artificial heavily modified water bodies where other surface waters include the transitional and coastal waters. The words 'not applicable' in the MAC-EQS column, means that the established AA-EQS values are also considered protective against short-term pollution.

*LOQ – limit of quantification = 30% of minimum EQS value for each pesticide. POPs – persistent organic pollutants.

2.3. The analytical performance of the LPME-based techniques

The development of precise, accurate and ultra-sensitive analytical methods, associated to simplicity and celerity, is still a hard task to undertake. In method development several parameters must be tested and optimized and numerous difficulties can be found especially in the sample preparation step. Recently, M. Dömötörova and Matisováa [52] made a review where the particularities and difficulties of pesticide analysis in different matrixes by chromatographic processes are mentioned. The choice of the solvent is pointed out as an important parameter when liquid-liquid extraction (LLE) is applied to sample preparation. LLE is a classic preconcentration technique that has been applied to a wide range of compounds [14, 15, 23, 53-55]. To overcome the shortcomings of LLE and to have simple, fast and green procedures microextractions techniques are being developed [26, 56]. Liquid phase microextraction (LPME) is a simple and low cost example. In LLE the analytes are extracted from an aqueous solution or donor phase into an organic solvent or acceptor phase. In a LPME the solvent can be a single microdrop suspended from a needle or it can be present in the pores of a hydrophobic membrane or separated from the donor phase by a membrane interface [57-59]. The dispersion of very fine droplets of organic solvents into the aqueous phase in a ternary solvent component system is another new option [26, 57]. As illustrated in Fig. 2.1, the LPME processes are generally divided into three main groups: a) single-drop microextraction (SDME); b) membrane liquid-phase microextraction (MLPME) and c) dispersive liquid-liquid microextraction (DLLME). Their differences rely in the way the solvent contacts the aqueous phase. As for solid microextractions, like SPME and SBSE, the analyses can be automated or not and can be carried out by direct immersion or by headspace in a static or dynamic mode in conjunction with other extractions techniques such as solid phase extraction (SPE) and supercritical fluid extraction (SFE). Based on these several characteristics many different configurations have been developed and some of them will be

focused below. More details concerning the extraction principles and the historical developments of LPME can be found in the recently review of Sarafraz-Yazdi and Amiri [57]. The chemical reactions involved in LPME and the developments of SDME have been pointed out by Li Xu's group [56, 60]. Moreover, the developments of hollow-fiber liquid-phase microextraction (HF-LPME) and its application in the analysis of environmental and biological samples are well described by the teams of Rasmussen [61] and Lee [62], respectively. The applications and progresses of DLLME can be found in the reviews of Ojeda [63], Rezaee [26] and Zang et al. [64].



DHF-LPME, dynamic hollow-fiber liquid-phase microextraction; DHS-THE-LPME, dynamic headspace time-extended helix liquid-phase microextraction; DHT-LPME, dynamic hook-type liquid-phase microextraction; IL-DLLME, ionic liquid dispersive liquid-liquid microextraction; DLLME, dispersive liquid-liquid microextraction; DLLME-LSC, Dispersive liquid-liquid microextraction with little solvent consumptions DLLME-SFO, solidification of a floating organic drop; HF-LPME hollow-fiber liquid-phase microextraction; HS-SDME, headspace single-drop microextraction; LLSME, liquid-liquid-solid microextraction; LPME, liquid-phase microextraction; MASE, membrane-assisted solvent extraction; MLPME, membrane liquid-phase microextraction; MMLLE, microporous-membrane liquid/liquid extraction; SDCME, single-drop coacervative microextraction; SDME, single-drop microextraction; SLME, supported-liquid membrane extraction; USAEME, ultrasound-assisted emulsification-microextraction.

Fig. 2.1 – Different configurations of LPME-based techniques applied to pesticide water analysis [26, 57-61].

2.3.1 Single-drop and membrane liquid-phase microextraction

Among the LPME-based techniques, SDME and MLPME were the first ones to be developed. Their differences relay in the way the acceptor phase is supported. In SDME the organic solvent contacts the aqueous phase through a suspended drop at the tip of a microsyringe. In MLPME the solvent can be present in the porosity of a membrane (HF-LPME) or it can be separated from the aqueous phase by a dense polymeric membrane (MASE). In 2007, Lambropoulou and Albanis [59] made a review of the application of SDME and MLPME to pesticides analysis in water samples. The optimization of LPME-based techniques as well as advantages and disadvantages were described. From their study, the authors conclude that the LMPE techniques have found many applications, the majority in environmental analysis. The review shows that a wide range of non-polar to moderate polar pesticides such as carbamates, chloroacetamide, chloroacetanilide, organochloride, organophosphorus, phenolic compounds, phenoxy acids, thiocarbamates and triazines have been extracted with SDME and MLPME from different matrices (water, soil and food) and analyzed by gas chromatography (GC) with mass spectrometry, electron capture or flame photometric detectors (GC-MS, GC-ECD, GC-FPD). High performance liquid chromatography (HPLC) with UV and MS detectors has also been used. The results also illustrate that for most of the non-priority pesticides analyzed in water samples the performance of the methods is near or in agreement with the published technical specifications. Since this last review new studies have been published. An example is the application of SDME by Pinheiro e Andrade Pinheiro et al. [65] in the analysis of organophosphorus and pyrethroids pesticides in water samples by SDME-GC-FID (flame ionization detector). An extraction of 30 min with 1 µL of toluene was sufficiently to achieve recoveries between 73 and 104%, however, the LOD of the method is in the range of 300 – 3000 ngL^{-1} higher than the values acceptable for drinking water. A mixture of organophosphorus and carbamates was also analyzed by

SDME couple with GC-MS and GC-NPD [66, 67]. Although the performances of the methods do not fulfill the European technical recommendations improvements were made in the detection by MS especially for malathion (organophosphate pesticide). The same SDME technique was also used by the group of C. Cortada [68] for the analysis of eighteen organochloride pesticides by GC-MS with selective ion monitoring (SIM) achieving limits of detection between 22 and 101 ngL^{-1} for extraction times of 37 min with 2 µL of toluene. These limits are well under the levels of sensitivity required by EPA methods. However, for the priority pesticides analyzed like aldrin, dieldrin, endosulfan, endrin, hexachlorocyclohexane (HCH) and *p,p'*-DDT, the LOD obtained do not comply with the statements of the WFD. In the study of Wang et al. [69] a fast run time was obtained in the determination of chlorophenols by SDME-GC-MS. During the preconcentration step, derivatization and extraction took place simultaneously. High extraction efficiency was found with a mixture of toluene and hexane (1:1). The derivatization was done 5% of *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MTBSTFA). Good recoveries (87.9 - 108.4%) and good precision (2.63 – 9.39%) together with low LOD were achieved (1.1 – 9.7 ngL^{-1}). For pentachlorophenol a LOD of 9.7 ngL^{-1} and a LOQ of 32.3 ngL^{-1} are considered a successful result. Comparing with other procedures, slightly lower LOD and LOQ were obtained under the same conditions when solid-phase microextraction (SPME) was used [69]. A less sensitive method was developed by Saraji and Bakhshi [70] when pentachlorophenol was analysed by GC-MS after extraction with hexyl acetate and derivatization with *N,O*-bis(trimethylsilyl)acetamide.

SDME is highly compatible with GC but with LC the sensitivity decreases as a consequence of the incompatibility of the organic solvents with LC mobile phase, where an extra step is needed for solvent evaporation and redissolution, and also as a consequence of small volumes used. To overcome these apparent difficulties a new method based on the application of

coacervates as extraction solvent of chlorophenols was developed by the research group of Lópes-Jiménez [71]. Coacervation is defined by IUPAC as the separation of colloidal systems into two liquid phases (one rich in colloid, i.e., the coacervate, and the other containing little colloid) [72]. Coacervates are thus colloid-rich liquids and seem to have unique properties to be adopted as solvents in SDME prior to LC analysis since they are non-volatile, which will restrict their evaporation, and can be compatible with LC mobile phases and with UV and MS detectors. Moreover, a larger volume drop can be used without disruption during the stirring of the solutions. In the work of Lópes-Jiménez [71] the application of decanoic acid vesicle-based coacervates for the analysis of pentachlorophenol by LC-UV the derivatization was eliminated which can be an advantage. In spite of the innovations, enforcements are still necessary since to improve the sensitivity of the method. It is possible that the research in this area and their application to the analysis of other class of pesticides will increase in the next few years since new processes of coacervation are being developed for the extraction of a wide range of compounds with different polarities which is still a drawback of the LPME-based techniques [72]. In spite of the several advantages of SDME like simplicity, low cost and speediness of the runs, the application of this technique to routine analysis is not yet a reality. Progresses in the automation have been made, however, the high cost involved probably will make the technique not widely accessible [60]. Meanwhile, the application of membrane liquid-phase microextractions is growing. In the recent times, organochloride pesticides were analyzed by Chen et al. [73] using a headspace HF-LPME technique followed by GC-MS. The compounds were extracted with 1-hexanol by dynamic hook-type liquid-phase microextraction (DHT-LPME) where a long polypropylene hollow fiber, hook shape, was suspended at the top of the sample vial. With this type of configuration it was possible to achieve a higher contact solvent surface for enrichment of the compounds. The method was optimized and validated. The recoveries were

found to be higher than 82% and the RSD was between 6.5 and 14% the LOD of 2 – 5 ngL^{-1} were near the recommended European values. Dieldrin, endosulfan, heptachlor, *o,p'*-DDT and *p,p'*-DDE were the substances analyzed. Based on this study and also on their experience in the application of a solvent cooling system to decrease the solvent loss during extraction of pesticides with hollow fibers, the same research group developed a method where the same organochloride pesticides, plus aldrin, were extracted with a dynamic headspace time-extended helix liquid-phase microextraction (DHS-TEH-LPME) prior to analysis by GC-MS/MS [74, 75]. A change in extracting solvent from 1-hexanol to 1-octanol, an increase in the fiber length´s and a decrease in the temperature of the extracting solvent improved significantly the LOD of the analytes, except for endosulfan. This type of extraction provided high enrichment factors along with excellent sample clean-up, however, as mentioned by the authors, the greatest weakness of the method was the unfeasibility to be fully automated and the poor precision obtained. HF-LPME was also applied to the analysis of carbamate pesticides by the researchers of Hylton [76] and Yang et al. [77]. The results obtained in both studies, with a HPLC-UV system and without derivatization of the compounds, showed that the LOD are well comparable to the LOD EPA methods which can be an indication that HF-LMPE is a promising tool for routinely pesticides analysis. To avoid the use of expensive microsyringes, the group of Berhanu [78] developed a new design of equilibrium in HF-LPME for the analysis of three organophosphorus pesticides (diazinon, chloropyrifos, and fenthion). In this new design the hollow fiber impregnated with *n*-undecane was connected to a copper wire and, at the end of the extraction time, the hollow fiber was removed with the help of the wire and soaked in ethyl acetate prior to GC-MS analysis. This type of configuration was found to be simple and of low cost. The LOD in reagent water using the GC-MS in the selected ion monitoring (SIM) were in the range of 15 - 80 ngL^{-1}. In spite of the good linearity and repeatability, for the priority substance

chloropyrifos, a LOD of 15 ngL^{-1} does not met the established performance criteria of the European legislation. In the study of Sanagi and Abidin [79] one triazole fungicide (hexaconazole) and two organophosphate insecticides (quinalphos and methidathion) were successfully analyzed by GC-ECD using toluene as HF-LPME solvent. The same insecticides quinalphos and methidation were investigated by Raharjo et al. [80]. The substitution of toluene by isooctanol and the separation and detection by HPLC-UV led to a considerably increase of the LOD. The same results were observed in the other study of Sanagi et al. [81] when a nylon cone shaped membrane was used to extract the organophosphates with hexane prior to their analysis by micro- LC-UV. Recently, Trtić-Petrović [82] provided a different method for the analysis of sixteen pesticides with different polarities by HPLC-MS/MS with electrospray ionization. In this study, some innovations were made relatively to the organic acceptor solvent. Initially, the compounds were extracted with *n*-hexyl ether but, to increase the extractions of the more polar pesticides the addition of a binary mixture of other solvents like tri-*n*-octylphosphine oxide (TOPO), dichloromethane, di(2-ethylhexyl) phosphoric acid (DEHPA) and tri-*n*-butyl phosphate (TBP) was made. The results show that better extraction efficiency was achieved when a solvent mixture of 10% of TOPO and 10% of TBP diluted in *n*-hexyl ether was used. For ten of the sixteen pesticides analyzed, the LOD ranged from 26 to 237 ngL^{-1} and the LOQ to 94 ngL^{-1} to 793 ngL^{-1}. Good linearity and precision, with RSD from 0.8 to 11.8%, was achieved for these ten compounds extracted at pH 8.0. Atrazine and diuron had an extraction efficiency of more than 80%. For diuron the LOQ is above the recommended value of 60 ngL^{-1}. Even though for the 6 more polar compounds the method performance was not validated, since the lowest concentration that could be analyzed was above the legislated value, enforcements are being made to overcome the difficulties found in the simultaneous extraction of pesticides having different chemical properties by LPME techniques.

The combination of solid-phase microextraction with liquid-phase microextraction has been an advance in HF-LPME especially for complex matrices. Hu et al. [83] developed a novel liquid–liquid–solid microextraction (LLSME) technique based on membrane molecular imprinted polymer microfiber to extract and concentrate the triazines which were further analyzed by HPLC-UV. In this technique, a membrane molecular imprinted polymer (MIP) coated silica micro-fiber was protected with a toluene filled polypropylene hollow-fiber membrane. With this type of configuration the analytes were first extracted to the organic phase and then adsorbed on the MIP coated silica microfiber. After the extraction the hollow membrane was removed and the analytes were desorbed from the microfiber by the usual procedure of SPME-HPLC commercial devices. Good precision, low LOD between 6 and 20 ngL^{-1} and acceptable recoveries (81.7 – 108.7%) in sludge water were obtained. This innovation appears to be a promising analytical tool for the monitoring of triazines in complex environmental waters since the target analytes can be selective separated from complex and dirty samples.

Membrane-assisted solvent extraction (MASE) has been another type of MPLME applied to pesticide analysis [59]. This technique was introduced in 2001 by Hauser and Popp [84] for the extraction of organochloride compounds in combination with large volume injection (LVI) gas chromatography electron capture detection (LVI-GC-ECD). In this study the use of a nonporous membrane was preferred to exclude any traces of water in the extracting solvent (heptane) which would adversely affected the injection system (LVI). The same research group worked on the extension of MASE to other environmental contaminates and developed a multiresidue/multi- class method for the analysis by LVI-GC-MS of 46 pesticides (30 organochlorides, 9 organophosphorous and 7 triazines) [85] extracted with 1 mL of cyclohexane. After the optimization of the extraction and detection conditions of the respective class of compounds, the fully automated MASE presented good precision with RSD less than 5% for organochloride and between 7 and 15%

for organophosphorous and triazines. The LOD were in the range of 2 - 10 ngL^{-1}. Dimethoate was an exception with a RSD of 24% and a LOD of 50 ngL^{-1}. For the most polar and water soluble compounds LLE was superior in terms of recoveries. Recently, van Pinxteren et al. [86] applied the same extraction technique for the analysis of 10 pesticides from different classes by HPLC–MS/MS using toluene as the acceptor phase. To compare the results, the compounds were extracted by the traditional SPE. Good precision, established by a RSD of 7 to 13%, was obtained with both extraction techniques. Acceptable recoveries (71 - 105%) were found with MASE. For the priority substances like atrazine, diuron, isoproturon and simazine a better sensitivity was achieved with MASE in compliance with the mandatory limits. For more volatile compounds like pentachlorophenol the technique is not the best option [87]. This technique shows potential to be a good alternative to the conventional off-line SPE for the analysis of low to medium polar compounds. As referred by the authors, the application of MASE to more polar compounds is still limited due to the non-polar character of the membranes. Another disadvantage can be the time of extractions which is higher when compared to other liquid-phase microextraction techniques. To the best of our knowledge no additional innovations were found with MASE in the field of pesticides water analysis. This type of technique seems to be more applied to very complex matrices like soils [59]. Anyway, the application of MASE will probably increase with the development of new suitable membranes especially for the extraction of more polar compounds. From the studies reviewed herein it is clear that the innovations in the SDME and MLPME techniques are growing and their application in the analysis of non-priority pesticides have been successful evolved. What concern the priority pesticides MLPME seems to be a promising preconcentration technique. As summarized in Table 2.2, for aldrin, dieldrin, endrin, isodrin, chlorofenviphos, DDT and HCH, improvements should be made to increase the sensitivity of the methods. For endosulfan, the results were still

substandard level. In the case of hexachlorobutadiene (HCB) and trifluralin no studies were found with reference to SDME or MLPME. For alachlor, atrazine, chloropyrifos, diuron, isoproturon and simazine the performance of the methods seem to fit the technical specifications for water pesticides monitoring.

Table 2.2 – The LPME based techniques applied to pesticides classified as priority substances and the performance of the associated methods. To compare the results the LOQ established by legislation were also included.

Priority Substance	Type of water	Type of LPME	Analysis	LOD (ngL^{-1})	Recovery (%)	RSD (%)	EF	Mandatory LOQ (ngL^{-1})	Ref.
Alachlor	Distilled water, river, lake, tap water	SDME	GC-ECD	**2.5**	88-102	6.8-7.9	-	**90**	[59]
	Milli-Q, drinking water	MASE	GC-MS (SIM)	**10**	72.3	10.7	-		[59]
Atrazine	Deionized water	HF-LPME	HPLC-MS/MS	**26**	-	4.3-8.9	-	**180**	[82]
	Water, sludge water	HF-LPME	HPLC-UV	**300**	-	-	-		[83]
	River, groundwater	MASE	HPLC-MS/MS	**1**	-	7	-		[86]
	Bidistilled water	MASE	LVI-GC-MS	**10**	-	9.1	-		[85]
	Water, sludge water	LLSME	HPLC-UV	**15**	94.3	3.1	-		[84]
	Water, tap, river water	DLLME	GC-MS	**60**		151	4.33		[92]
	Ultrapure, tap, reservoir groundwater	DLLME	HPLC-UV	**600**	99.5	6.4	-		[93]
Chlorfenvinphos	Ultrapure water	SDME	GC-NPD	**200**	-	-	-	**30**	[67]
Chloropyrifos	Farm, river, well water	SDME	GC-FPD	**4**	-	8.6-13.4	552	**9**	[59]
	Ultrapure water	SDME	GC-NPD	**800**	-	-	-		[67]
	Ultrapure water	HF-LPME	GC-MS (SIM)	**15**	-	14.8	-		[76]
	Doubly-distilled, tap, well, rain water	DLLME (IL)	HPLV-UV	**5000**	101.8-113.4	2.4-4.0	-		[125]
Cyclodienes								**1.5**	
Aldrin	Water, tap, reservoir water	SDME	GC-ECD	**20**	90.9	3.7	20		[59]
	Water, wastewater	SDME	GC-MS	**53**	90 ± 8	9.9	-		[68]
	Rainwater	HF-LPME	GC-MS (SIM)	**59**	79.3-98.7	2.01	105		[59]
	Water and seawater	HF-LPME	GC-MS (SIM)	**6**	-	8.6	-		[59]

	Seawater	HF-LPME	GC-MS (SIM)	**59**	83.6-89.5	2.01	105	[59]
	Water, river water	DHS-THE-LPME	GC-MS/MS	**0.33**	106 ± 17	15.4	1112	[74]
	Bidistilled water	MASE	LVI-GC-MS	**5**	-	10.9	-	[85]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**7**	-	5.4	708	[112]
	Tap, lake water	DLLME-SFO	GC-ECD	**21.6**	90.2-94.8	8.5	37	[111]
	Distilled, river tap, surface water	DLLME	GC-MS	**9**	81-97	7	-	[96]
	River, reservoir water	DLLME-LSC	GC-MS (SIM)	**0.6-1.2**	93.9-104.5	4.1-7.1	-	[102]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	90-98	-	-	- [106]
	Water, tap, reservoir water	SDME	GC-ECD	**5**	96.7-96.1	5.7	60	[73]
	Water, wastewater	SDME	GC-MS	**53**	90 ± 8	9.9	-	[59]
	Water, wastewater	SDME	GC-MS	**22**	78 ± 8	6.3	-	[68]
	Water and seawater	HF-LPME	GC-MS (SIM)	**1**	-	5.7	-	[59]
	Rainwater	HF-LPME	GC-MS (SIM)	**47**	74.9-87.3	2.32	92	[59]
	Seawater	HF-LPME	GC-MS (SIM)	**47**	91.4-97.1	2.32	92	[59]
	Water, river water	DHS-THE-LPME	GC-MS/MS	**25**	106 ± 12	16.3	1184	[74]
Dieldrin	Deionized, rainwater	DHT-LPME	GC-MS	**2**	84.2	14.4	328	[73]
	Deionized, rainwater	Static-LPME	GC-ECD	**8.1**	-	15.86		[73]
	Bidistilled water	MASE	LVI-GC-MS	**2**	-	7.3	-	[85]
	Distilled, river tap, surface water	DLLME	GC-MS	**4**	82-100	9	-	[95]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**19**	-	6.4	1290	[112]
	River, reservoir water	DLLME-LSC	GC-MS (SIM)	**0.4-1.1**	93.6-100.5	8.2-8.9	-	[102]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	83-94	-	-	- [106]
	Water, wastewater	SDME	GC-MS	**68**	78 ± 6	9.8	-	[68]
Endrin	Water and seawater	HF-LPME	GC-MS (SIM)	**8**	-	4.7	-	[59]

	Rainwater	HF-LPME	GC-MS (SIM)	**33**	85.6-93.1	1.93	92	[59]
	Rainwater	HF-LPME	GC-MS (SIM)	**31**	82.9-102.2	5.50	69	[59]
	Seawater	HF-LPME	GC-MS (SIM)	**33**	77.3-93.7	1.93	98	[59]
	Seawater	HF-LPME	GC-MS (SIM)	**31**	89.3-90.0	5.50	69	[59]
	Bidistilled water	MASE	LVI-GC-MS	**2**	-	7.0	-	[85]
	Distilled, river tap, surface water	DLLME	GC-MS	**4**	81-102	8	-	[96]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**14**	-	7.2	1337	**-** [112]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	94-103	-	-	**-** [106]
Isodrin	Bidistilled water	MASE	LVI-GC-MS	**10**	-	8.1	-	[85]
DDT total (1)								**7.5**
	Water, tap, reservoir water	SDME	GC-ECD	**200**	90.5-92.6	9.6	55	[59]
	Water, wastewater	SDME	GC-MS	**101**	50 ± 10	7.8	-	[68]
	Rainwater	HF-LPME	GC-MS (SIM)	**17**	81.6-97.6	1.66	68	[59]
	Seawater	HF-LPME	GC-MS (SIM)	**17**	81.7-94.7	1.66	68	[59]
	Water, seawater	HF-LPME	GC-MS (SIM)	**1**	-	7.4	-	[59]
***p,p'*-DDT**	Bidistilled water	MASE	LVI-GC-MS	**2**	-	9.5	-	**3** [85]
	Distilled, river tap, surface water	DLLME	GC-MS	**4**	75-93	11	-	[96]
	Water, melted snow, river water	DLLME	HPLC-UV	**320**	95.67-110.0	4.10	100	[95]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**16**	-	5.5	1190	[112]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	75-83	-	-	[106]
	Water, river water	DHS-THE-LPME	GC-MS/MS	**0.37**	98 ± 15	17.4	1520	[74]
***o,p'*-DDT**	Deionized, rainwater	DHT-LPME	GC-MS	**4**	94.4	6.5	370	[73]
	Bidistilled water	MASE	LVI-GC-MS	**2**	-	7.9	-	[85]
	Tap, lake water	DLLME-SFO	GC-ECD	**25.1**	85.1-90.0	8.8	181	[111]

	Water, melted snow, river water	DLLME	HPLC-UV	**510**	91.00-106.2	2.80	100		[95]
***p,p′*-DDE**	Water, tap, reservoir water	SDME	GC-ECD	**50**	94.2-98.3	5.4	55		[59]
	Water, wastewater	SDME	GC-MS	**25**	43 ± 5	9.0	-		[68]
	Water and seawater	HF-LPME	GC-MS (SIM)	**1**	-	10.6	-		[59]
	Water, river water	DHS-THE-LPME	GC-MS/MS	**0.27**	104 ± 15	17.8	2121		[74]
	Deionized water, rainwater	DHT-LPME	GC-MS	**4**	99.6	10	445		[73]
	Bidistilled water	MASE	LVI-GC-MS	**2**	-	4.7	-		[85]
	Water, melted snow, river water	DLLME	HPLC-UV	**350**	86.56-119.6	7.50	100		[95]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**10**	-	6.3	986		[112]
	Tap, lake water	DLLME-SFO	GC-ECD	**28.3**	86.3-102.5	7.2	872		[111]
	Distilled, river tap, surface water	DLLME	GC-MS	**2**	81-99	7	-		[96]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	98-100	-	-	**-**	[106]
***p,p′*-DDD**	Water, wastewater	SDME	GC-MS	**22**	47 ± 7	6.9	-		[68]
	Rainwater	HF-LPME	GC-MS (SIM)	**28**	85.0-108.4	2.28	67		[59]
	Seawater	HF-LPME	GC-MS (SIM)	**28**	92.1-95.2	2.28	67		[59]
	Water and seawater	HF-LPME	GC-MS (SIM)	**1**	-	7.4	-		[59]
	Bidistilled water	MASE	LVI-GC-MS	**2**	-	6.8	-		[85]
	Water, melted snow, river water	DLLME	HPLC-UV	**40**	85.58-103.5	6.13	100		[95]
	Distilled, river tap, surface water	DLLME	GC-MS	**4**	84-96	8	-		[96]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**8**	-	4.9	884		[112]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	95-100	-	-	**-**	[106]
Diuron	Deionized water	HF-LPME	HPLC-MS/MS	**64**	-	5.2-9.6			[82]
	River and groundwater	MASE	HPLC-MS/MS	**0.5**	-	9	-	**60**	[86]

	Deionized, river, tap well water	DLLME	HPLC-DAD	**70**	89.108	6.4	60		[104]
Endosulfan [2]	Rainwater	HF-LPME	GC-MS (SIM)	**28**	79.4-90.1	3.13	155	**0.15**	[59]
	Seawater	HF-LPME	GC-MS (SIM)	**28**	92.0-93.3	3.13	155		[59]
α-endosulfan	Water, tap and reservoir water	SDME	GC-ECD	**200**	83.3-90.4	4.6	70		[18]
	Ultrapure, tap, surface water	SDME	GC-ECD	**10**	90-100	1.7-5.5	4.9		[59]
	Water, wastewater	SDME	GC-MS	**64**	47 ± 6	7.6	-		[68]
	Water, river water	DHS-THE-LPME	GC-MS/MS	**19**	109 ± 15	12.5	633		[74]
	Deionized water, rainwater	DHT-LPME	GC-MS	**5**	85.8	12.2	275		[73]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**16**	-	5.5	1267	**-**	[112]
	Tap, lake water	DLLME-SFO	GC-ECD	**12.1-19.7**	87.0-96.2	7.6	808		[111]
	Distilled, river tap, surface water	DLLME	GC-MS	**5**	83-95	6	-		[96]
	River and reservoir water	DLLME-LSC	GC-MS (SIM)	**0.4-0.8**	99.5-102.0	5.6-9.7	-		[102]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	94-101	-	-	**-**	[106]
β-endosulfan	Ultrapure, tap, surface water	SDME	GC-ECD	**10**	90-100	4.9	-		[59]
	Water, wastewater	SDME	GC-MS	**71**	52 ± 5	7.9	-		[68]
	Bidistilled water	MASE	LVI-GC-MS	**10**	-	7.4	-		[85]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**9**	-	5.9	1091	**-**	[111]
	Tap, lake water	DLLME-SFO	GC-ECD	**12.9**	85.5-93.5	5.8	286		[111]
	Distilled, river tap, surface water	DLLME	GC-MS	**25**	85-103	15	-		[96]
	River, reservoir water	DLLME-LSC	GC-MS (SIM)	**1.3-2.5**	93.1-101.6	6.0-8.2	-		[102]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	90-101	-	-	**-**	[106]
Hexachlorobenzene	Milli-Q, drinking water	MASE	GC-MS (SIM)	**0.02**	47.3	2.6	-		[59]
	Reagent water, groundwater	MASE	LVI-GC-ECD	**10**	93	9	-	**30**	[84]
	Reagent water	MASE	LVI-GC-MS	**2**	-	6.0	-		[85]

Hexachlorobutadiene	Water, tap, lake water	DLLME-SFO	GC-ECD	**3**	93-98	8.7	219	**30**	[110]
	Water, tap, lake water	DLLME-SFO	GC-MS	**45**	100-102	1.3	283		[110]
Hexachlorocyclohexane[3]:								**0.6**	
α-HCH	Water, wastewater	SDME	GC-MS	**87**	103 ± 8	6.7	-		[68]
	Rainwater	HF-LPME	GC-MS (SIM)	**17**	86.1-106.7	13.72	139		[59]
	Seawater	HF-LPME	GC-MS (SIM)	**17**	91.8-93.6	13.72	139		[59]
	Water, seawater	HF-LPME	GC-MS (SIM)	**1**	-	6.6	-		[59]
	Milli-Q, drinking water	MASE	GC-MS (SIM)	**0.01**	81.8	15.5	-		[59]
	Deionized, river, drinking water	MASE	GC-MS (SIM)	**10-25**	107.6	5.2	-		[59]
	Reagent water, groundwater	MASE	LVI-GC-ECD	**10**	89	6	-		[21]
	Bidistilled water	MASE	LVI-GC-MS	**5**	-	5.7	-		[85]
	Distilled, river, tap, surface water	DLLME	GC-MS	**3**	101-113	7	-		[96]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	100-103	-	-		[106]
β-HCH	Water, wastewater	SDME	GC-MS	**93**	100 ± 8	6.5	-		[68]
	Rainwater	HF-LPME	GC-MS (SIM)	**29**	87.4-111.6	10.29	83		[59]
	Seawater	HF-LPME	GC-MS (SIM)	**29**	85.3-91.3	10.29	83		[59]
	Water and seawater	HF-LPME	GC-MS (SIM)	**5**	nr	5.5	nr		[59]
	Milli-Q, drinking water	MASE	GC-MS (SIM)	**0.02**	73.8	16.7	-		[59]
	Reagent water, groundwater	MASE	LVI-GC-ECD	**10**	-	-	-		[84]
	Bidistilled water	MASE	LVI-GC-MS	**10**	-	4.8	-		[85]
	Distilled, river, tap, surface water	DLLME	GC-MS	**5**	96-112	25	-		[95]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	98-100	-	-	**-**	[106]

γ-HCH	Water, tap, reservoir water	SDME	GC-ECD	**20**	97.2	3.2	95	[59]
	Water, wastewater	SDME	GC-MS	**45**	102 ± 8	6.5	-	[68]
	Rainwater	HF-LPME	GC-MS (SIM)	**13**	93.4-112.6	14	74	[59]
	Seawater	HF-LPME	GC-MS (SIM)	**13**	84.1-86.52	14	74	[59]
	Water, seawater	HF-LPME	GC-MS (SIM)	**3**	-	6.5	-	[59]
	Milli-Q, drinking water	MASE	GC-MS (SIM)	**0.04**	98.5	16.0	-	[59]
	Reagent water, groundwater	MASE	LVI-GC-ECD	**10**	93	9	-	[84]
	Bidistilled water	MASE	LVI-GC-MS	**10**	-	6.0	-	[85]
	Distilled, river tap, surface water	DLLME	GC-MS	**8**	96-111	5	-	[96]
	Deionized, tap, river, agriculture water	DLLME-SFO	GC-ECD	**11**	-	5.8	1311	[112]
	Ultrapure, tap water	USAEME	GC-MS	**21**	103-109	6-9	-	[105]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	100-103	-	-	[106]
δ-HCH	Water, wastewater	SDME	GC-MS	**66**	101 ± 8	8.2	-	[68]
	Water, seawater	HF-LPME	GC-MS (SIM)	**2**	-	5.5	-	[59]
	Milli-Q, drinking water	MASE	GC-MS (SIM)	**0.01**	68.7	17.1	-	[59]
	Reagent water, groundwater	MASE	LVI-GC-ECD	**25**	105	8	-	[84]
	Bidistilled water	MASE	LVI-GC-MS	**2**	-	5.4	-	[85]
	Distilled, river, tap, surface water	DLLME	GC-MS	**6**	86-109	8	-	[96]
	Distilled, tap, well, surface water	USAEME	GC-μECD	**2-16**	100-102	-	-	[106]
Pentachlorophenol	Purified, river water	SDME	GC-MS	**61**	-	9.3-10.7	138	**120** [70]
	Distilled, river water	SDME	GC-MS	**9.7**	94.7	4.61	-	[69]

	Distilled, superficial, reservoir, groundwater	SDCME	HPLC-UV	**300**	92-105	4.8	-	[71]
	Reagent water, groundwater	MASE	LVI-GC-MS	**595**	95.4	12.7	-	[87]
	Reagent water, groundwater	MASE	LVI-GC-ECD	**10**	93	9	-	[84]
	Water, tap, well, river water	DLLME	GC-ECD	**10**	98.7-101.3	2.1-2.4	710	[90]
	Water, tap, mineral, river water	LMPE-SFO	GC-MS	**5**	89.2-93	8.3	1035	[113]
Isoproturon	River and groundwater	MASE	HPLC-MS/MS	**0.5**	-	7	-	**90** [87]
	Water, river water	PDLLME	HPLC-UV	**100-280**	91-104	1.5-5.9		[103]
Simazine	Deionized water	HF-LPME	GC-MS (SIM)	**10**	94.3-104.5	0.78-2.68	190	[59]
	Deionized water	HF-LPME	HPLC-MS/MS	**61**	-	3.6-10.8	-	[83]
	Surface water	SLME	HPLC-UV	**100**	85	-	-	[59]
	Deionized water	MASE	GC-MS (SIM)	**5-100**	-	10.4	-	**300** [59]
	River, groundwater	MASE	HPLC-MS/MS	**2.5**	-	10	-	[87]
	Bidistilled water	MASE	LVI-GC-MS	**10**	-	7.2	-	[86]
	Water, tap, river water	DLLME	GC-MS	**120**	109.7-115.9	151	4.31	[93]

(1) DDT total comprises the sum of the isomers: p,p'-DDT; *o,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD; (2) Endosulfan is a mixture of two stereoisomers: α-endosulfan or endosulfan I comprises 64-67% of the tech. grade and β-endosulfan or endosulfan II, comprises 29-32% tech. grade [88]; (3) Hexachlorocyclohexane (HCH) is a mixture of four stereoisomers: α-HCH, β-HCH, γ-HCH, δ-HCH. For material containing ≥ 99% of γ-HCH the common name is lindane [88].

2.3.2 Dispersive liquid-liquid microextraction

Dispersive liquid–liquid microextraction (DLLME) is another recent technique that has been successfully applied to the extraction and concentration of a wide variety of pesticides from water samples. DLLME was developed in 2006 by Rezaee and co-workers [26, 89] and is based on a ternary solvent component system involving an aqueous phase, a non-polar water immiscible solvent (extracting solvent) and a polar water miscible solvent (disperser solvent). In this technique, fine droplets of the extracting solvent are dispersed into the aqueous phase when an appropriate mixture of both solvents is injected into water samples. Following mixing a cloudy solution is formed and after centrifugation or solidification after cooling, the fine particles of the extracting solvent containing the target analytes are separated from the aqueous phase. High recoveries and high enrichment factors can be reached and the extraction time can be relatively short. The mixing of the three components ensures equilibration within a few seconds due to the large interface between the multiple fine extractor droplets and the aqueous solution. DLLME can be regarded as a multiple drop microextraction. Water insoluble and high density extracting solvents have been mostly used. Chlorobenzene, chloroform, carbon disulfide and carbon tetrachloride are some examples [26]. Acetone, acetonitrile, methanol and ethanol have been the main options as dispersive solvents. DLLME can be coupled with GC, HPLC and also with atomic absorption spectrometry (AAS) [26]. The non-selective characteristic of the extraction solvents can be sometimes a disadvantage. To overcome this difficulty and to eliminate the use of dispersive solvents, making the technique more environmental friendly, new alternatives are being developed like the use of ionic liquids (IL) and ultrasonic radiation. Due to its simplicity and low extraction time, DLLME is becoming an attractive preconcentration technique in pesticide water analysis. This extraction technique has been efficiently applied by Fattahi et al. [90] in the analysis of chlorophenols by GC-ECD. As

before, derivatization and extraction were done simultaneously. Within a few seconds both steps were complete and after centrifugation the analytes were prepared to be injected into the GC. For pentachlorophenol recoveries of 98.7 – 101.3%, relative standard deviations (RSD) of 2.1 – 2.4% and a LOD of 10 ngL^{-1} were achieved. Equivalent results were obtained by Wang et al. [69] in the analysis by SDME-GC-MS. Since the recommended LOQ for pentachlorophenol is 120 ngL^{-1}, this means that the methods developed can be a competitive option to the standard methods applied for such classes of compounds that use the classic non-green LLE such as EN ISO 12673:1998 [91] or EPA 8270D:1998 [92]. Another group of pesticides, with LOD between 46 and 120 ngL^{-1}, analyzed by DLLME with GC-MS was the triazine herbicides [93]. Atrazine was also investigated by Zhou et al. [94] using a less sensitive method based on DLLME-HPLC-UV. For this class of compounds better sensitivity was achieved in the study of Hu et al. [83] when a membrane liquid-phase microextraction (MLPME) was used in the preconcentration step. Zhou et al. [95] also developed a method based on the same extraction technique to quantify the famous banned organochloride insecticide (*p,p'*-DDT) and its main metabolites (*p,p'*-DDE, *p,p'*-DDD) by HPLC-UV. In spite of the acceptable values for the method repeatability and extraction efficiency the LOD are above the EQSs. The authors compared their method with other methods based on solid (SPE and SPME) as well as on liquid-phase microextractions (SDME and HF-LPME) and found out that for the compounds under investigation, the most sensitive was the SPE-HPLC-UV method (LOD of 4 – 13 ngL^{-1}). Supported on their experience on SDME, the group of Cortada [68, 96] investigated the performance of the analysis of eighteen organochloride pesticides by DLLME-GC-MS. Considerable improvements were made in the method sensitivity when DLLME was applied relatively to SDME. The LOD of the compounds was more close to the LOD imposed by recent legislation. For DDT and its metabolites the DLLME-GC-MS method performance is comparable with the one developed by Zhou et al. [97] where SPE-HPLC-UV was used to quantify

the same compounds. Like chlorophenols, organochloride and triazine herbicides, carbamates were also extracted by DLLME. In the study of Liu et al. [98] the pesticides were extracted with chloroform using acetonitrile as dispersive solvent. In spite of the good recoveries, linearity and precision, the LOD of the DLLME-HPLC-DAD method was in the μgL^{-1} range. A slightly different method was used by He et al. [99] in the analysis of the same class of pesticides. Similar outcomes were obtained when chlorobenzene was used as extracting solvent. Caldas et al. [100] analyzed by DLLME coupled with LC–ESI-MS/MS three different classes of pesticides, namely, the carbamate carbofuran, clomazone and tebuconazole. Acceptable recoveries (62.7 – 120%) associated to good precision (RSD between 1.9 - 9.1%) and low LOD (20 ng/L) meet the requirements for their determination in water samples. In the work of Fu et al. [101] the use of DLLME with HPLC-FLD (fluorescence detector) was successfully applied in the simultaneous analysis of one carbamate pesticide (carbaryl) and one organophosphorous insecticide (triazophos) in waters and fruit juices. Good precision and recoveries of 80 – 114% and LOD ranging from 12.3 to 16.0 ngL^{-1} were well achieved. In the study of Tsai and Huang [102] a new adaptation of the DLLME was investigated. Dispersive liquid–liquid microextraction with little solvent consumptions (DLLME-LSC) was applied to the extraction of priority substances aldrin, dieldrin, endosulfan and heptachlor from river, tap and seawater. The separation and detection were done by GC-MS and the results were acceptable for river and tap water. A high enrichment factor and high recoveries (90.5 – 109.4%) were achieved in the extraction step of the compounds using as extraction solvent a mixture of tert-butyl methyl ether and tetrachloroethylene (6:4). Associated to these results are good repeatability and sensitivity (LOD of 0.4 – 2.5 ngL^{-1}) which makes the method a promising for routine analysis. Another DLLME configuration, named partitioned dispersive liquid–liquid microextraction (PDLLME), was studied by Chou et al. [103] in the analysis of the phenylurea herbicides by HPLC-UV. Although the authors agree that in PDLLME the extraction efficiency of polar

compounds seems to increase, since the polar compounds, depending on their partition coefficients, can be extracted into the dispersive solvent (tetrahydrofuran) as well as into the extracting solvent (dichloromethane), the sensitivity of the method is still beyond the desirable European values. Better results were obtained in the work of Saraji and Tansazan [104], especially for linuron, when the conventional DLLME was applied using acetone and carbon disulfide as dispersive and extracting solvent, respectively. An innovative configuration of DLLME based on ultrasound-assisted emulsification-microextraction (USAEME) has been developed by Regueiro et al. [105] for the analysis of emergent contaminates and pesticides in environmental waters by GC-MS. Musk fragrances, phthalate esters and lindane (γ-HCH) were the compounds under investigation. With USAEME the mass transfer process between the aqueous phases and the extraction solvent is accelerated by ultrasonic radiation speeding up the extraction efficiency and the time of the analysis (10 min). Although the LOD is at the ngL^{-1} level for most of the compounds, for lindane (γ-HCH) a LOD of 21 ngL^{-1} and a LOQ of 71 ngL^{-1} does not meet the requirements recommended by the WFD. The same extractive technique was applied by Ozcan et al. [106] in the analysis of eighteen organochloride pesticides by gas chromatography with micro-electron capture detection (GC-μECD) achieving LOD between 2 and 16 ngL^{-1}. The compounds evaluated were hexachlorocyclohexane (4 isomers), heptachlor epoxide, dieldrin, aldrin, endrin, endrin aldehyde, endrin ketone, endosulfan (2 isomers), endosulfan sulphate, *o,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD and methoxychlor. The method seems to be more selective for lindane than the one developed by Regueiro et al. [105], nevertheless, the sensitivity for all the compounds is still below the limits imposed by the EC Directives. Ultrasound-assisted emulsification-extraction was also investigated by Feo et al. [107] in the analysis of fourteen pyrethroid insecticides by gas chromatography with negative ion chemical ionization mass spectrometric detection using ammonia as reagent gas (GC-NCI-MS). Acceptable recoveries (63 – 105%) and good repeatability with

RSD of 2 – 5% were achieved using 1 mL of chloroform as extracting solvent. The LOD of 0.03 to 35.8 ngL^{-1} makes the procedure a potential method for pyrethroids insecticides water monitoring. Another class of pesticides that has been isolated by ultrasound-assisted microextraction was the carbamates. In the work developed by Wu et al. [108] a surfactant (Tween 20) was used to accelerate the ultrasound emulsion formation decreasing the compounds extraction time. The separation and detection was done by HPLC-DAD and the recoveries in the spiked river, reservoir and well water were satisfactory. The authors compared the precision and sensitivity of their method with other earlier studies and found out that the LPME-based techniques showed better sensitivity in comparison to solid-phase microextraction technique (SPME) [76, 109]. The results also show that when the new DLLME technique was applied the extraction time was significantly reduced from 120 min with SPME to 3 min with DLLME.

To overcome the problems associated to the use of highly toxic solvents in the usual process of DLLME, new procedures are being published using low density organic compounds which can be further solidified to increase the precision and accuracy of the methods. The group of Leong et al. [110] applied the technique of solidification of a floating organic drop (SFO) with DLLME in the analysis, by GC-ECD and GC-MS, of some halogenated organic compounds like 1,2-dichlorobenzene, 1,2,4-trichlorobenzene, tetrachloroethylene, hexachlorobutadiene and 4-bromodiphenyl ether. Better LOD was obtained with the electron capture detector. For hexachlorobutadiene a LOD of 3 ngL^{-1} can be an acceptable value since its mandatory LOQ is ten times higher. Organochloride pesticides were also analyzed with the same method by the same research group [111]. In spite of the good recoveries (82.9 – 102.5%) and precision (RSD of 5.8 – 8.8%), the LOD of heptachlor, aldrin, α-endosulfan, β-endosulfan, *o,p*-DDT and *p,p*'-DDE stood between 11 and 110 ngL^{-1} well above the recommended limits. The same organochloride compounds, plus dieldrin, endrin, lindane, *p,p*'-DDD and *p,p*'-DDT, were evaluated by Farahani et al. [112]

using a similar method. A change in the extracting solvent from hexadecane to 1-dodecanol and an increase of the temperature and extraction time led to a better precision and lower LOD (7 – 19 ngL^{-1}) for most of the eleven pesticides analyzed. SFO combined with DLLME was also used by Faraji et al. [113] in the investigation of phenolic compounds where the toxic pentachlorophenol was included. Once more, derivatization and extraction were done in one step. Acetic anhydride and 1-undecanol were sequential mixed with water samples and after approximately 17 min the organic solvent was solidified in an ice bath, transferred to a clean vial and finally injected in the GC-MS. Precision and recoveries were lower than the method applied by Fattahi et al. [90] but the sensitivity was improved especially for pentachlorophenol. Extractions with 1-undecanol followed by drop solidification were also used by the group of Khalili-Zanjani [114] in the quantification of organophosphorous pesticides by GC-FPD. The results suggest that the method seems to be less sensitive than the one applied by Berijani et al. [115] who used the traditional high solvent density DLLME procedure achieving LOD in the ngL^{-1} range. A slightly different method was presented by Farajzadeh et al. [116] in the determination of the same class of pesticides by GC-FID and GC-MS. In this study cyclohexane was used as low density extracting solvent. Better LOD, in the range of ngL^{-1}, was achieved with mass spectrometric detection. The results were similar to ones by Berijani et al. [115]. Chen et al. [117] also used the same low density microextraction solvent based technique in the analyses of 4 carbamates by GC-MS/MS. An extraction time of 10 min with toluene and acetonitrile as dispersing solvent provided, in conjunction with the detection system, good repeatability with RSD in the range of 2.7 – 6.8%, efficient recoveries (94.5 – 104%) and better LOD (1 – 50 ngL^{-1}) than the method of Hylton [76] and Wu et al. [109].

In DLLME the association with solid-phase extractions techniques is becoming a usual procedure. In the work of Zhao et al. [118] chloroacetanilide herbicides were extracted and concentrated by SPE-DLLME prior to GC-MS

injection. The herbicides were adsorbed from a large volume of water samples onto a multi-walled carbon nanotube, eluted with acetone and then concentrated by DLLME. With this hyphenated technique a better precision was achieved when compared with the work of Zhao et al. [119] where SDME was used to extract the same compounds. In both studies the LOD are below the European recommendations. The combination of stir bar sorptive extraction (SBSE) and DLLME has been another approach made by the group of Farajzadeh [120] for the determination of triazole fungicide by GC-FID and GC–MS. For hexaconazole the method seems more precise and sensitive than the one applied by Sanagi et al. [81] which was based on a MLPME extraction. Another advance in DLLME has been the use of ionic liquids (IL). Their immiscibility in water and the high capacity to dissolve organic compounds make them suitable for analytical extraction purposes [121-125]. Some applications have been made to pesticide analysis by HPLC-UV, but the LOD is above the environmental limits [126-129]. An exception was published by Xie et al. [130] in the analysis of three organophosphorous pesticides (phorate, parathion, and phoxim) where LOD between 1 and 10 ngL^{-1} was obtained using the IL 1-butyl-3-methylimidazolium hexafluorophosphate as extracting solvent ([C4MIM][PF6]). Until now the most applied IL in pesticide analysis has been 1-octyl-3-methylimidazolium hexafluorophosphate ([C8MIM][PF6]) and 1-hexyl-3-methylimidazolium hexafluorophosphate ([C6MIM][PF6]). It is quite sure that advances will be made in this area. The application of other IL and the synthesis of new ones specially designed for the analysis of multiple classes of pesticides will be an option in the next years. Moreover, as column contamination by IL is the main difficulty found in GC analysis, it is likely that in the future the GC and LC analytical system will be design to overcome such problems. From the data published one can see that the main advantage of DLLME seems to be the low extraction time which is sometimes associated to good method performance. With this type of preconcentration technique improvements were made in the analysis of some of the priority pesticides.

Aldrin, dieldrin, endrin, α-endosulfan, and β-endosulfan are examples when USAEME was used. As before, no data was found for the analysis of trifluralin by DLLME. Among all the LPME techniques summarized in Table 2.2, MASE seems to be associated to more sensitive methods for the described pesticides, except for alachlor (SDME), aldrin, *o,p'*-DDT and *p,p'*-DDE (HF-LPME).

2.4. Futures perspectives

From the studies related herein it is clear that LPME-based techniques seem to be a potential extraction procedure for the analysis of pesticides in water samples. Several organic solvents have been used and more options will appear in the future. The application of the LPME-based techniques to standard multiresidue/multiclass methods will probably be a reality soon. In the study of Mamun et al. [15] a method for the screening of 82 multiclass pesticides by GC with electron capture detection (GC-ECD) was developed based on the extraction procedures. Recoveries from water samples were found to be between 82% and 120% and the LOD ranged from 20 to 2000 ngL^{-1}. Since the extraction of the compounds was done by LLE with dichloromethane it is expected that in the future the application of LPME-based techniques will be an alternative to LLE in standard analytical procedures [18, 19, 21, 92]. Moreover, the association of LPME-based techniques to modern chromatographic methods like gas chromatography high resolution mass spectrometry (GC-HRMS), fast GC and two-dimensional gas chromatography (GC×GC) and ultra high performance liquid chromatography (UHPLC) will be most likely a powerful option for the development of rapid ultra sensitive analytical methods. Moreover, the isolation of the target analytes with hyphenated extraction techniques seems to be an advance especially for complex matrices. It is possible that soon this type of devices will appear as an integral part of the actual hyphenated chromatographic and detection systems.

2.5. Conclusions

Pesticides are present in everyday life of any community. Depending on their toxicity, the acceptable values imposed by European legislation in water samples are mostly at trace and ultra-trace levels. To have low LOD and LOQ in compliance with the technical specifications especial attention has been paid to the extraction and concentration of the target analytes. All authors agree that LPME-based techniques are simple, time-saving and of low cost when compared to other microextraction techniques. Several methods have been developed based in the methodologies of SDME, MLPME and DLMME. For some priority pesticides like alachlor, aldrin, atrazine, chloropyrifos, DDT and its metabolites, dieldrin, diuron, hexachlorobenzene, hexachlorobutadiene, isoproturon, pentachlorophenol and simazine the LPME-based techniques offer a valid and reliable alternative to the well established but non green LLE. For endrin, isodrin, hexachlorocyclohexane (HCH) and especially endosulfan future efforts shall be addressed to increase methods sensitivity. For the other non priority pesticide, the LPME-based techniques are promising tool to develop very sensitive analytical methods in compliance with the established threshold limits set up by the European Community.

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3

Effects of Ultrasonic Irradiation and Direct heating on the Extraction of Priority Pesticides from Marine Sediments

Highlights

- Multi-frequency ultrasonic baths are a promising tool in the analytical field.
- The application of ultrasounds and direct heating reduced the extraction cycles.
- To avoid degradation a compromise between intensity and frequency must be met.
- Sediment screening can give information about pesticides fate and water quality.
- Method performance was in good agreement with quality control guidelines.

❖ M. I. Pinto, G. Sontag, C. Vale, J.P. Noronha, ''Effects of ultrasonic irradiation and direct heating on extraction of priority pesticides from marine sediments'', **Int. J. Environ. Anal. Chem.**, 93 (2013), 1638-1659 (DOI 10.1080/0306319.2013.831409).

Abstract

Priority pesticides (alachlor, aldrin, γ-chlordane, chlorfenvinphos, chlorpyrifos, dieldrin, *p,p'*-DDT, *p,p*-DDD, *p,p'*-DDE, α-endosulfan, β-endosulfan, endosulfan sulphate, endrin, α-HCH, β-HCH, γ-HCH, δ-HCH, HCB, HCBD, heptachlor, heptachlor epoxide, isodrin, methoxychlor, mirex, quintozene, terbuthylazine and trifluralin) are a group of toxic substances that are known by their persistency in the aquatic environment. Their screening in marine sediments may provide information on the sources and distribution in the water mass of fresh transitional and coastal waters. This work proposes a rapid and reliable method to extract multi-residues of priority pesticides by ultrasounds irradiation from marine sediments. Multiple variables have been optimized: ultrasound frequency, sonication intensity, signal operation mode, time of extraction and water bath temperature. After sample clean-up and pesticides preconcentration by stir bar sorptive extraction, the compounds were analyzed by gas chromatography-mass spectrometry (GC-MS) using the selective ion monitoring acquisition mode (SIM). Better performance was found to ultrasonic-assisted extractions (UAE) at frequency of 35 kHz and an output intensity of 60% in a sweep mode of operation. An increase of water bath temperature to 80 ºC had a significant effect on the extraction of pesticides with high octanol-water partitioning coefficients (K_{ow}). Under optimal conditions, method detection limits (MDLs) and method quantification limits (MQLs) ranged from 0.3 to 4.4 ngg^{-1} and from 0.8 to 14 ngg^{-1}, respectively. Recoveries between 70 and 111%, at high precision levels, were found at different types of marine sediments with a single extraction cycle. Method performance was in good agreement with quality control guidelines.

Keywords: Gas Chromatography-mass spectrometry (GC-MS); Marine sediments; Multi-frequency ultrasonic baths; Priority pesticides; Ultrasound-assisted extraction (UAE).

3.1. Introduction

Ultrasound-assisted extraction (UAE) is a simple and low-cost sample preparation technique widely applied to food and soil analysis [1, 2]. UAE involves the use of shear forces created by sound waves at frequencies above the 20 kHz [3]. Cavitational activities occurred at the surface of solid particles and the acoustic vortex microsteaming formed within the pores of solid particles are thought to be major factors responsible for compounds extraction [4, 5]. In simple terms, cavitation is the growth and the collapse of preexisting microbubbles under the influence of an ultrasonic field in liquids [6]. Extremely high localized temperatures and pressures around the 5000 K and 2000 atm, respectively, are generated during bubbles collapse [5]. When in contact with solid surfaces, bubbles implosion produces high-speed microjets and high-pressure shockwaves causing surface erosion and particle fragmentation [2]. Depending on the ultrasonic frequency highly reactive hydroxyl radicals may be formed during cavitation course [2]. The formation of this type of radicals may change the surface chemistry by oxidation processes and ultimately may degrade the target compounds [2]. All referred ultrasound physicochemical effects improve solvent diffusivity, analytes solubility and mass transport. Their extension and subsequent extraction efficiency are dependent on the characteristics of the ultrasonic energy. The UAE can be performed with ultrasonic probes or with ultrasonic baths, ultrasonic probes delivering higher ultrasonication intensity than ultrasonic baths [7]. However, the probability of compounds degradation is much higher and the number of samples that can be sonicated at once is lower. The main disadvantage of usual ultrasonic baths is the lack of uniform distribution of the ultrasound energy [7, 8]. In common ultrasonic baths, the irradiation conditions are constant and unchangeable. To overcome this limitation and to improve their performance the most advanced ultrasonic baths incorporate several features where better extraction conditions can be achieved by optimization of the different parameters such as the frequency,

sonication intensity, the signal operation mode and the water bath temperature [7]. The application of this type of tunable multi-frequency ultrasonic baths is relatively new in the analytical field and few articles were found concerning pesticides analysis [7, 8].

Investigations about the effects of the ultrasonic energy variables on the extraction of compounds are still scarce and the application of UAE to the determination of pesticides in marine and estuarine sediments has not been much explored in as with soil samples [1, 3, 5, 9]. Priority pesticides are an important group of persistent compounds, listed as priority hazardous substances under the Annex X of the Water Framework Directive [10] and also as Persistent Organic Pollutant (POPs) under Stockholm Convention [11]. Screening of the priority pesticides in sediments can provide information on the sources, distribution and bioavailability. All these aspects are crucial to assess the water quality.

In view of that, the aim of the present study was to evaluate and to optimize the effects of the ultrasonic conditions, generated by a tunable multi-frequency ultrasonic bath, on the development of a multi-residue analytical method used for the screening of priority pesticides (alachlor, aldrin, γ-chlordane, chlorfenvinphos, chlorpyrifos, dieldrin, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, α-endosulfan, β-endosulfan, endosulfan sulphate, endrin, α-HCH, β-HCH, γ-HCH, δ-HCH, HCB, HCBD, heptachlor, heptachlor epoxide, isodrin, methoxychlor, mirex, quintozene, terbuthylazine and trifluralin) in marine sediments samples. Though terbuthylazine is not listed as a priority substance it was included due to its relevance in coastal ecosystems. Sample clean-up and pesticides preconcentration was optimized by stir bar sorptive extraction (SBSE) prior to gas chromatography-mass spectrometry (GC-MS) analysis. To improve compounds sensitivity the selective ion monitoring (SIM) acquisition mode was selected (GC-MS/SIM).

3.2. Materials and methods

3.2.1. Reagents and standard solutions

Acetonitrile was purchased from Carlo Erba (Rodano, Italy). Acetone, *n*-hexane, toluene and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride was obtained from Panreac (Barcelona, Spain). A mixture of aldrin, dieldrin, endrin, α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH, δ-HCH, *p,p'*-dichloro-diphenyltrichloroethane (*p,p'*-DDT), *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), α-endosulfan, β-endosulfan, endosulfan sulphate, heptachlor, heptachlor epoxide and methoxychlor was obtained from Supelco (EPA organochloride pesticide mix n.º46960-U, Bellefonte, PA, USA) at individually concentrations of 2000 μgmL^{-1} in *n*-hexane:toluene (1:1). γ-Chlordane (100 μgmL^{-1}) in *n*-hexane was also purchased from Supelco (Bellefonte, PA, USA). Alachlor, bromopropylate (surrogate), chlorfenvinphos, chlorpyrifos, hexachloro-1,3-butadiene (HCBD), hexachlorobenzene (HCB), isodrin, mirex, quintozene, terbuthylazine and trifluralin were purchased from Sigma-Aldrich (St. Louis, MS, USA) with a high purity level (97.0 – 99.9%). These individual standards were prepared at a concentration of 2000 μgmL^{-1} in *n*-hexane or *n*-hexane:acetone (2:1) depending on their solubility in organic solvents. A mixture of all the pesticides at a concentration of 10 μgmL^{-1} was prepared by appropriate dilution of the stock standards solutions in *n*-hexane:acetone (9:1). Triphenylmethylchloride was obtained from Sigma-Aldrich (St. Louis, MS, USA) and used as internal standard (IS). It was prepared at a concentration of 2000 μgmL^{-1} in *n*-hexane:acetone (2:1) and further diluted to 5 μgmL^{-1} in *n*-hexane:acetone (9:1). The pesticide *p,p'*-DDT used during quality control tests was purchased from Sigma-Aldrich (St. Louis, MS, USA). A solution at a concentration of 100 μgmL^{-1} was prepared in toluene and diluted to 1 μgmL^{-1} in *n*-hexane:acetone (9:1). All the solutions were kept at 4 ºC and protected from light. Sample clean-up and analytes pre-concentration was carried out

with a 20 mm × 0.5 mm (length × film thickness) polydimethylsiloxane (PDMS) coated twister stir bar (50 µL PDMS volume) supplied by Gerstel GmbH (Mülheim, Germany). To avoid effects of carryover after each sorption step, stir bars were left in DMSO for 60 min at 100 rpm. Prior to use, stirs bars were conditioned in acetonitrile for 30 min in the ultrasonic bath. Spiking of sediments samples was made in accordance the procedure published by K. L. Smalling et at. [12]. The standard reference material (SRM) 1941b (organics in marine sediments) was obtained from National Institute of Standards and Technology (Gaithersburg, USA).

3.2.2. Equipment

The chromatographic equipment consisted of an Agilent Technologies 6850 GC Network System with an Agilent 5975C VL MS detector (Agilent Technologies, Waldbronn, Germany). The ultrasonic extractions were done with high-performance multi-frequency ultrasonic bath (100 W, 35 kHz/130 kHz) from Elma Transonic TI-H-5 (Singen, Germany). Centrifugation was carried out on a Sigma Laboratory Centrifuge 4K15 (Buckinghamshire, UK).

3.2.3. Sampling

Surface sediments samples (approximately 5 cm thickness) were collected in the Óbidos Lagoon located on the west coast of Portugal. In the laboratory, samples were dried at room temperature during 48 h, homogenized, and sieved with a 2 mm mesh sieve device to remove large fractions such as stones and gravel [13, 14].

3.2.4. Sample preparation

Sample preparation consisted of two steps: **1**. Extraction of the pesticides from the marine sediment samples by ultrasonic-assisted extraction (UAE) and **2**. Sample clean-up and pesticides preconcentration by stir bar sorptive extraction (SBSE).

3.2.4.1 Ultrasonic-assisted extraction

3.2.4.1.1 Frequency, output intensity, standard and sweep mode of operation

15 ± 0.01 g of sediment sample spiked with 70 ngg^{-1} of each pesticide were placed in a 50 mL polypropylene tube. 20 mL of acetonitrile were added and the extractions were performed at 35 kHz and 130 kHz at room temperature. For each of these two frequencies an output intensity of 60% and 100% was studied. The extractions were carried out for 10 min in a standard and sweep operation mode. After extraction, the samples were centrifuged for 5 min at 1986 RCF. 15 mL of the extract were further transferred to a volumetric flask for sample clean-up and analytes enrichment by SBSE.

3.2.4.1.2 Sample amount, extraction volume, extraction time and temperature

A sample of 10 or 15 ± 0.01 g of sediment sample spiked with 70 ngg^{-1} of each pesticide were placed in a 50 mL polypropylene tube. 10, 15 or 20 mL of acetonitrile were added. The extractions were performed for 10, 20 and 30 minutes at a frequency of 35 kHz and an intensity output of 60% in the sweep mode of operation. The extractions were carried out at room temperature, 60 °C and 80 °C. The solutions were further centrifuged at 1986 RCF for 5 min. Depending on the extraction volume, 5, 10 or 15 mL of extract were transferred to a volumetric flask for sample clean-up and analytes enrichment by SBSE.

3.2.4.1.3 Optimized conditions for ultrasonic-assisted extraction

10 ± 0.01 g of sediment sample were placed in a 50 mL polypropylene tube. 10 mL of acetonitrile were added and the extractions were performed at 35 kHz with an output intensity of 60% in a sweep operation mode at 80 ^{o}C for a period of 30 min. The solutions were then centrifuged at 1986 RCF for 5 min to carry on the SBSE step.

3.2.4.2 Sample clean-up by SBSE

3.2.4.2.1 Extraction volume, effect of salting out, sorption time, stirring rate, desorption

15 mL of the extract were diluted to 30, 50, 75 and 100 mL with Milli-Q water. 5 or 10 g of sodium chloride were added when extracts were diluted to 100 mL to evaluate the effect of salting out. When UAE was carried out with 10 or 15 mL of acetonitrile, sample clean-up was performed with 5 and 10 mL of sample extract, respectively. During sample clean-up acetonitrile concentration was kept constant at 15% (v/v). After sample dilution, a stir bar coated with PDMS was placed in the diluted samples and the sorption was carried out for 2, 3 and 16 hours (overnight) at room temperature and at 300 and 900 rpm. The stir bar was then removed, rinsed, gently dried in a tissue paper and placed in a graduated tube with 2 mL of *n*-hexane:acetone (9:1) or ethyl acetate. The desorption of the analytes was performed for a period of 15 min at 200 rpm and 500 rpm and also for a period of 30 min at 200 rpm The stir bar was removed and the solution was evaporated to 0.5 mL under a gentle stream of nitrogen. 2 µL were injected into the GC-MS system.

3.2.4.2.2 Optimized conditions for sorption and desorption

5 mL of the extract were diluted with 85 mL of Milli-Q water plus 10 mL of acetonitrile. Sorption was carried out with a PDMS stir bar for a period of 16 hours (overnight) at room temperature and at 300 rpm. After sorption, the stir bar was removed, rinsed, gently dried in a tissue paper and placed in a graduated tube with 2 mL of *n*-hexane:acetone (9:1). Analytes desorption was carried out as described in 3.2.4.2.1 (15 min, 200 rpm). All experimental tests were made in duplicate.

3.2.5. Analysis by GC-MS/SIM

The pesticides were separated on a silica capillary column (30 m x 0.25 mm i.d.; d_f: 0.25 µm) covered with 5% phenyl and 95% of dimethyl-

polysiloxane (HP-5MS, Agilent-J&W Scientific) at a helium flow rate of 1 $mLmin^{-1}$. For mass spectrometric (MS) detection the ion source, the transference line and the detector temperature were kept at 230 ºC, 150 ºC and 290 ºC, respectively. The MS spectra were obtained by Electronic Impact (EI) at 70 eV using Agilent ChemStation software. GC injection parameters were: 2 µL of injected solution in a pulsed split less mode (solvent delay 3 min); injector temperature 280 ºC. GC temperature: 65 ºC, 18 ºC min^{-1} to 150 ºC isothermal 12 min, 3 ºC min^{-1} to 225 ºC isothermal 2 min, 18 ºC min^{-1} to 260 ºC isothermal 6.4 min. The mass spectrometric detector (MSD) was operating in the full-scan aquisition mode and the SIM mode.

3.2.6. Internal quality control

The internal quality control of the results was made by running blanks, spiked samples (recovery) and samples in duplicate (precision and assessment of sample heterogeneity and matrix effects). An evaluation of the *p, p´*-DDT breakdown was used to check the liner performance. Liners that are contaminated, chemically active or too hot can cause compound degradation (breakdown) especially when *p, p´*-DDT and endrin are among the target analytes [15]. The percentage of *p,p´*-DDT breakdown was calculated by Equation (1):

$$\%DDT_{breakdown} = \frac{(DDD + DDE)}{DDT_{Total}} * 100 \quad (\mathbf{1})$$

where, DDD = *p,p'*-DDD peak area; DDE = *p,p'*-DDE peak area; DDT_{Total} = Total DDT peak area (*p,p'*-DDT + *p,p'*-DDD + *p,p'*-DDE) [16, 17]. To evaluate the quality of the results acceptance criteria were established in accordance with the quality control guidelines as: **a**) blanks with no interferences; **b**) precision ≤20%; **c**) recoveries in the range of 70 - 120% and **d**) *p,p'*-DDT breakdown ≤20% [15, 18, 19].

3.3. Results and discussion

3.3.1. Standard solution

The retention times and the ions used for identification, confirmation and subsequent quantification are presented in Table 3.1. Three ions of *m/z* >100 are the minimum data required when SIM mode is used for pesticides analysis [18, 19]. For quantification the selected ion was the one that showed no evidence of chromatographic interference, best signal-to-noise ratio and higher abundance.

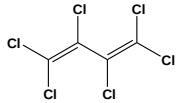
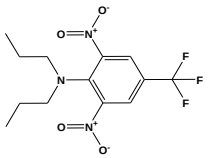
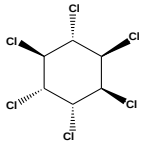
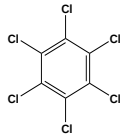
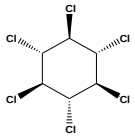
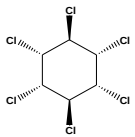
3.3.2. Sediment samples

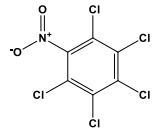
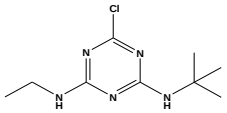
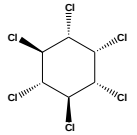
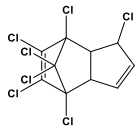
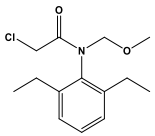
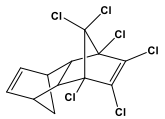
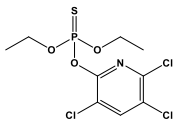
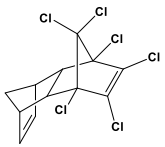
After extraction and SBSE, mass spectral confirmation was based upon retention times and the ratios of the integrated areas for selected ions to the integrated area of the most abundant of the selected ions being tracked. The established criterion was that each ratio (as a percentage) should correspond to that observed for a calibration standard to within 10 unities [18, 19, 23]. The percentage of relative abundance was calculated with respect to the most abundant ion from each standard. Using trifluralin as an example, the ratio of the peak area (at 17.01 min) from *m/z* = 264 to that of the peak area at *m/z* = 306 (most abundant peak) needs to be between 70% and 90% while that of the ratio of peak at *m/z* = 335 to that of the area of the peak at *m/z* = 306 needs to be between 4% and 24% [19, 23].

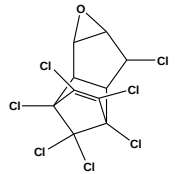
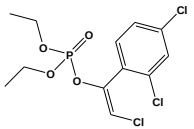
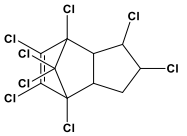
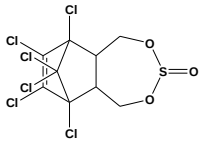
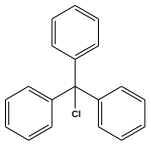
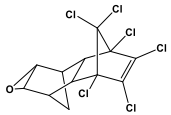
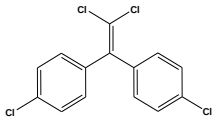
3.3.2.1 Ultrasonic-assisted extraction

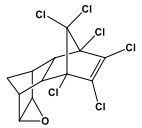
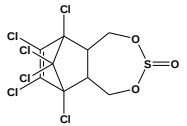
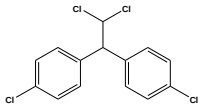
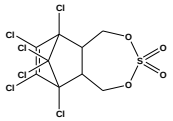
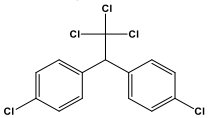
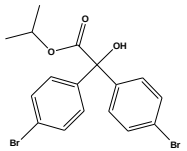
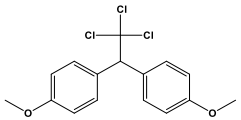
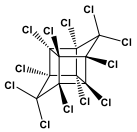
The influence of different parameters on the extraction of the target pesticides from marine sediments was investigated to find out the optimal conditions for their extraction.

Table 3.1 – Mass spectrometry data for GC-MS/SIM analysis of priority pesticides and some physico-chemical properties (M is the molecular weight, RT is the retention time and K_{ow} the octanol-water partitioning coefficient) [18-22].

Compound Name CAS[a]	Chemical Structure	M ($gmol^{-1}$)	Log K_{OW}	RT (min)	SIM[b, c] (*m/z*)
Hexachloro-1,3-butadiene 87-68-3		260.76	4.78	6.21	190 (46%), 225 (100%), 260 (35%)
Trifluralin 1582-09-8		335.28	5.34	17.01	264 (80%), 306 (100%), 335 (14%)
α-Hexachlorocyclohexane (α-HCH) 319-84-6		290.83	3.81	17.56	146 (25%), 181 (100%), 219 (77%)
Hexachlorobenzene 118-74-1		284.78	3.55	18.23	142 (40%), 249 (29%), 284 (100%)
β-Hexachlorocyclohexane (β-HCH) 319-85-7		290.83	3.81	20.36	146 (38%), 181 (100%), 219 (84%)
γ-Hexachlorocyclohexane (γ- HCH or Lindane) 58-89-9		290.83	3.55	20.79	146 (25%), 181 (100%), 219 (77%)

Quintozene 82-68-8		295.34	4.64	21.28	249 (80%), 265 (100%), 294 (60%)
Terbuthylazine 5915-41-3		229.7	3.21	21.92	173 (55%), 214 (100%), 229 (40%)
δ-Hexachlorocyclohexane (δ-HCH) 319-86-8		290.83	4.14	23.16	146 (40%), 181 (100%), 219 (80%)
Heptachlor 76-44-8		373.32	3.87 – 6.10	26.83	237 (50%), 272 (100%), 337 (30%)
Alachlor 15972-60-8		269.77	3.09	27.50	160 (100%), 188 (82%), 238 (35%)
Aldrin 309-00-2		364.91	5.17 – 7.40	29.40	255 (30%), 263 (100%), 293 (40%)
Chlorpyrifos 2921-88-2		350.59	3.31 – 5.27	30.73	197 (100%), 208 (35%), 314 (53%)
Isodrin 602-050-00-4		364.91	5.20 – 5.45	31.49	147 (35%), 193 (100%), 263 (43%)

Heptachlor epoxide 1024-57-3		389.32	3.65 – 4.43	32.45	237 (30%), 263 (30%), 353 (100%)
Chlorfenvinphos 470-90-6		359.57	3.10 – 4.22	33.80	267 (100%), 295 (25%), 323 (54%)
γ-Chlordane 57-74-9		409.8	2.78	34.15	237 (30%), 266 (15%), 373 (100%)
α-Endosulfan 959-98-8		406.93	4.74	34.91	195 (100%), 237 (80%), 339 (35%)
Triphenylmethyl chloride 76-83-5 *Internal Standard*		278.77	--	36.35	154 (30%), 183 (100%), 260 (20%)
Dieldrin 60-57-1		380.91	5.20	36.68	263 (100%), 279 (65%), 380 (30%)
p,p'-DDE 72-55-9		318.03	6.00	37.04	176 (45%), 246 (100%), 318 (70%)

Endrin 72-20-8		380.91	5.20	38.02	243 (50%), <u>263</u> (100%), 281 (57%)
β-Endosulfan 33213-65-9		406.93	4.79	38.72	<u>195</u> (100%), 237 (70%), 339 (30%)
p,p'-DDD 72-54-8		320.04	5.87	39.74	165 (50%), 199 (16%), <u>235</u> (100%)
Endosulfan sulphate 1031-07-8		422.92	-	41.54	229 (70%), 237 (70%), <u>272</u> (100%)
p,p'-DDT 50-29-3		354.49	6.79	42.04	165 (56%), 199 (20%), <u>235</u> (100%)
Bromopropylate 18181-80-1		428.12	5.4	45.45	155 (30%), 183 (60%), <u>341</u> (100%)
p,p'-Methoxychlor 72-43-5		345.65	4.95 - 5.08	45.89	165 (10%), 212 (7%), <u>227</u> (100%)
Mirex 2385-85-5		545.55	5.28	46.88	<u>237</u> (100%), 271 (11%), 332 (20%)

[a] CAS number – registered number at the Chemical Abstract Service; [b] quantification ions are underlined; [c] Percent relative abundance with respect to the most abundant ion from standards are between parentheses.

3.3.2.1.1 Ultrasound frequency, operation mode and output intensity

A common trend was observed when the experimental tests were done at a constant frequency of 35 kHz and in the sweep mode of operation. As shown in Fig. 3.1a, for *p,p´*-DDT, trifluralin, chlorpyriphos and α-HCH at 35 kHz peak areas decreased when intensity output increased from 60% to 100%. The same drift was observed in the standard operation mode but with some exceptions for β-HCH, terbuthylazine, δ-HCH, chlorpyriphos, chlorfenvinphos, dieldrin, endrin, β-endosulfan, *p,p'*-DDD and bromopropylate. For these pesticides equal or slightly high peak areas were obtained when intensity output increased from 60% to 100% as illustrated for three of them in Fig. 3.1b.

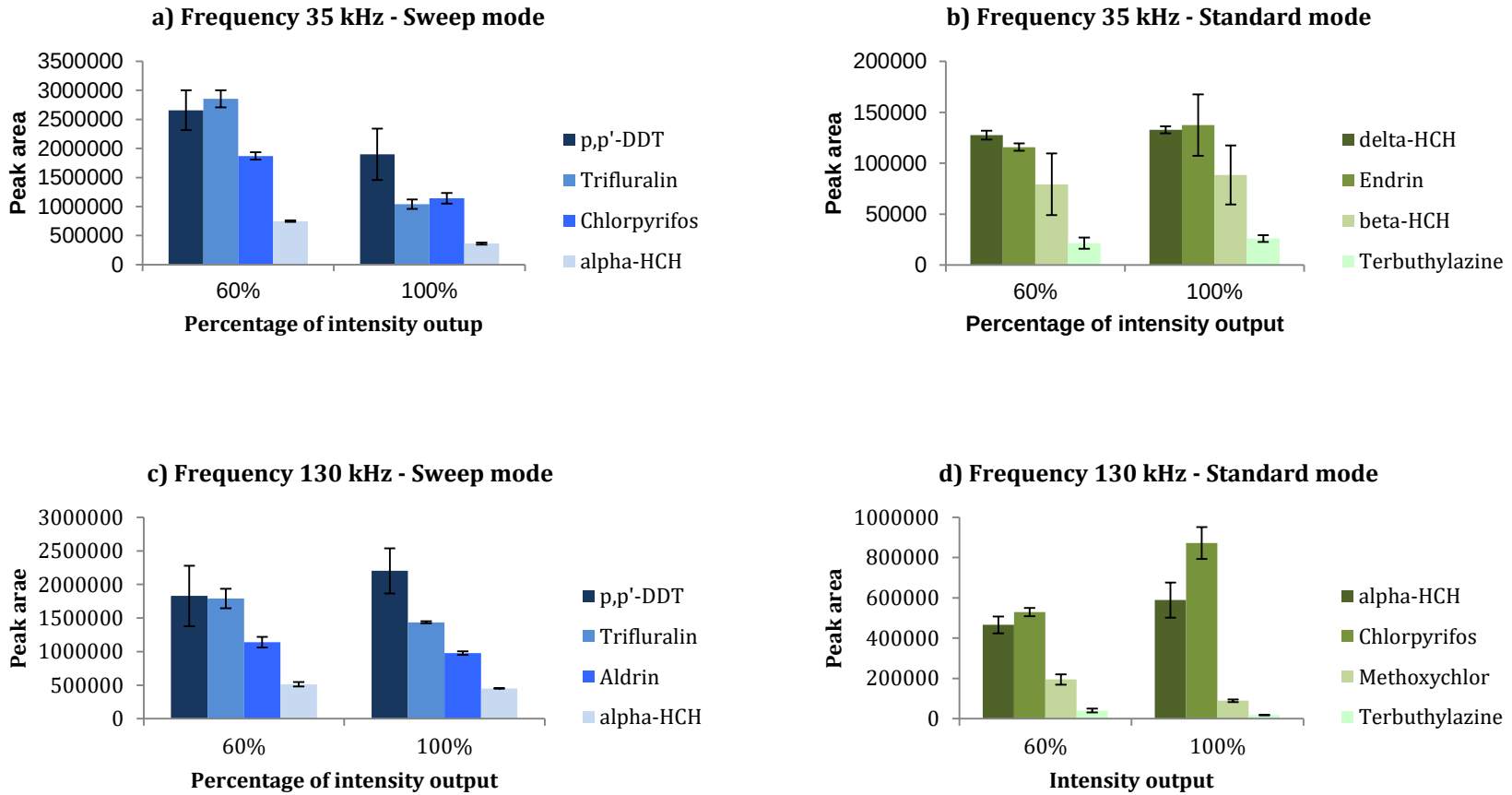


Fig. 3.1 – Influence of the intensity output (%) on the extraction of the priority pesticides: (**a**) 35 kHz in the sweep mode of operation; (**b**) 35 kHz in the standard mode of operation (with drift exceptions); (**c**) 130 kHz in the sweep mode of operation; (**d**) 130 kHz in the standard mode of operation. Results are presented as the mean ± standard deviation.

Conversely, at a higher frequency of 130 kHz and under the sweep mode of operation, peak areas increased when the intensity output varied from 60% to 100% (illustrated for *p,p'*-DDT in Fig. 3.1c). Hexachloro-1,3-butadiene (HCBD), trifluralin, α-HCH, hexachlorobenzene, δ-HCH, quintozene, heptachlor, alachlor, aldrin, chlorpyriphos, bromopropylate and mirex showed an opposite tendency (Fig. 3.1c). Under the same frequency (130 kHz) and in the standard mode of operation, peak areas also increased when the intensity output increased from 60% to 100% (shown for α-HCH and chlorpyrifos in Fig. 3.1d). However, the number of exception was much lower when compared with the sweep mode of operation. Among the studied pesticides, only methoxychlor and terbuthylazine did not follow the same directly tendency between frequency and intensity (Fig. 3.1d). When comparing the two modes of operation, the results reveal that the number of exceptions was lower at lower frequencies (35 kHz) under the sweep mode of operation, while under the standard mode the variability was lower at higher frequencies (130 kHz). In the standard operation mode the frequency is normally regulated against the mechanical resonance of the ultrasound transducer while in the sweep mode the signal sent to the transducer varies (sweeps) slightly in frequency over a set period of time. With this type of sweep operation mode the standing wave moves up and down within the tank and the energy is equally distributed across the water bath [24]. In sweep mode ultrasounds distribution was more effective at lower frequencies.

The comparison of all the obtained data shows higher peak areas at the frequency of 35 kHz with an intensity output of 60% in the sweep mode of operation. Exceptions were chlorfenvinphos and methoxychlor, since higher peak areas were found at 130 kHz with a full intensity output (100%) in the sweep and standard mode, respectively. The results obtained in this work are in line with the findings reported by G. Cravotto et al. [25] and D. Chen et al. [3]. As the frequency of the irradiation increases, the rarefaction phase

shortens and consequently it is necessary to increase the intensity (power) to maintain an equivalent amount of cavitational energy in the medium. In fact, when the total intensity given by the equipment was used (100%), higher peak areas were obtained at a higher frequency (130 kHz) whereas at 60% of the intensity output the extraction efficiency was higher when a lower frequency of 35 kHz was applied. A similar cavitational activity seems to be generated when working with the full intensity (100%) at higher frequencies, or when working with the less intensity power (60%) at lower frequencies (35 kHz). To reach maximum extraction values there is a compromise between the intensity and the frequency necessary. Although the cavitation effects increase with the increase of the intensity and the decrease of the frequency, from the analytical point of view this might not be the optimal conditions for extraction procedures. High intensities and low frequencies lead to high sonication effects, which can promote some undesired consequences such as analyte degradation [2, 16, 17]. It should not be discard the possibility that, at 35 kHz under full intensity output, the studied pesticides have been degraded by the cavitation effects, and consequently the peak areas decreased. Indeed, R. Rial-Otero et al. [8] found out that the cavitation effects produced by the ultrasonic bath at 35 kHz and an intensity output of 100% were enough to decompose the acaricide amitraz in acidic medium at room temperature. These authors reported high recoveries at the frequency of 130 kHz using the ultrasonic bath in the sweep mode of operation for the analysis of bromopropylate and coumaphos in honey samples while, for fluvalinate the standard mode was a better option [8]. In the current study, the extraction of bromopropylate from sediments was higher when the sweep mode was used at 60% of the intensity output and at frequency of 35 kHz. The same behavior was observed for most of the priority pesticides under analysis, and therefore, those were the conditions used on method validation.

3.3.2.1.2 Extraction time

Peak areas increased from 3 to 45% when sonication time increased from 10 to 20 min. Hexachloro-1,3-butadiene, trifluralin, γ-HCH, hexachloro-benzene, aldrin, chlorpyrifos, α-endosufan, dieldrin and bromopropylate showed a peak area increase between 15 to 18% when the extraction time changed from 20 to 30 min, whereas methoxychlor had an opposite behavior showing a signal decrease of about 8%. Therefore, a sonication time of 30 min was selected as the optimum time for the extraction since higher values were found for the majority of the pesticides.

3.3.2.1.3 Extraction temperature

Ultrasound-assisted extraction (UAE) was carried out at room temperature, 60 ºC and 80 ºC which is the maximum temperature of the ultrasonic bath. An extraction at 80 ºC without ultrasonic energy was also carried out. As shown in Fig. 3.2, the application of ultrasounds had a significant effect on mirex response. An increase of 40% was found. However, increments on water bath temperature did not improve mirex extraction since equivalent peak areas were obtained under sonication effects.

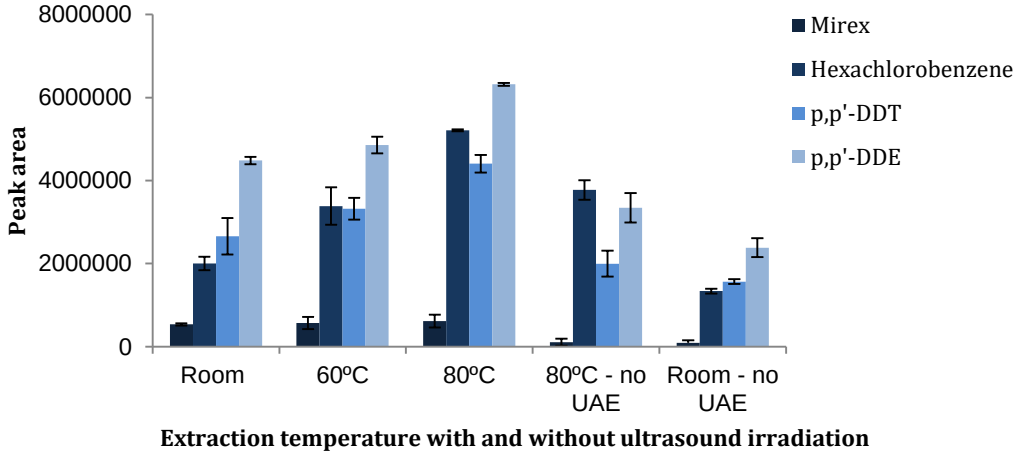


Fig. 3.2 – Influence of the extraction temperature on the extraction of the priority pesticides (temperature profile). Results are presented as the mean ± standard deviation.

Apart from mirex, the application of ultrasounds at high temperatures (80 ºC) had a considerable impact on the extraction of some other compounds as illustrated in Fig. 3.2 by hexachlorobenzene, *p,p'*-DDT and its metabolite *p,p'*-DDE. The difference between peak areas obtained at 80 ºC, with and without UAE, was higher for *p,p'*-DDT, *p,p'*-DDE and aldrin, which are the studied pesticides with the highest values of $\log k_{ow}$ (Table 3.1). An increase in the range of 46 to 95% in peak areas was registered for these compounds. The other pesticides with lower $\log k_{ow}$ presented increments between 10 to 40%. As can been seen from Fig. 3.2, hexachlorobenzene showed higher peak areas at 80 ºC without UAE than at sonication temperature of 60 ºC. The same behavior was found for hexachloro-1,3-butadiene, trifluralin, quintozene, chlorpyriphos and chlorfenvinphos. Temperature seems to play an important role in the extraction of these compounds. For all quantified pesticides lower peak areas were found when extractions were carried out at room temperature without ultrasounds.

Variation of temperature is one of the simplest methods of modifying cavitation activity [26]. A change in the temperature entails a change in a number of parameters that influence formation, growth and bubbles collapse, having a direct consequence on compounds extraction. The combination of high temperatures (50 - 90 ºC) with ultrasonic energy has been applied mainly to metal ion analysis probably due to their thermal stability and also to some pharmaceutical compounds and endocrine disrupters in sewage sludge [5, 27-32]. The majority of the works point out a decrease in the cavitation activity with an increase of temperature [5]. However, some contradictory data has been reported [26]. As pointed out by N.V. Deshkunov [26] depending on the irradiation conditions the cavitational activity may decrease as temperature increases or may increase reaching a maximum. This last situation was observed by N.V. Deshkunov [26] at low ultrasonic intensities and at frequencies of 21.9 kHz and 880 kHz. Although, the mechanisms involved in the ultrasonic extraction is beyond the scope of this

work, the results reveal that the combination of ultrasounds irradiation, at low frequencies and low intensities, with temperatures near the boiling point of acetonitrile (82 ^{o}C), have a positive effect on pesticides extractability especially for those that tend to bind tightly to soil and sediments.

3.3.2.2 Sample clean-up and pesticides preconcentration by SBSE

Sample clean-up and pesticides preconcentration was carried out in one single step by SBSE. Analytes were isolated from the diluted acetonitrile extract by sorption into magnetic stir bars coated with polydimethylsiloxane (PDMS). The effect of extract dilution, salt addition (salting-out), time of sorption and stirring rate were tested as well as desorption time and the best solvent to be used in pesticides back extraction prior to GC-MS analysis. SBSE is a simple and green analytical technique that has been insufficiently explored in the determination of pesticides in marine sediments [33, 34].

3.3.2.2.1 Extract dilution and effect of salting-out

To increase the affinity of the pesticides towards the stir bars PDMS-phase the acetonitrile extracts were diluted with Milli-Q water. High peak areas were found when a final volume of 100 mL was used, except for mirex where better results were found at 75 mL. For a final volume of 100 mL, the addition of 5 g and 10 g of sodium chloride did not improve peak area responses, except for chlorfenvinphos and terbuthylazine. For these pesticides better results were found at 10% (w/v) of sodium chloride.

3.3.2.2.2 Sorption time and stirring rate

Sorption tests conducted for different time and stirring rates reveal that for a period of 2 hours an increase in the stirring rate from the 300 rpm to 900 rpm did not improve pesticides sorption. Stirring rates above 900 rpm were excluded because of the instability of the stir bar. Higher sorption was obtained for a period of 3 hours at 900 rpm but with higher standard deviations. Bubble formation might be the reasons of such poor precision results [35, 36]. To improve the sensitivity a longer time of 16 hours

(overnight) was tested at 300 rpm. High peak areas were found at those specific conditions for all pesticides. Similar sorption time results were achieved by other authors who have extracted organochloride pesticides from water, soil and sediments samples by SBSE using the same type of stir bars [33, 37-39]. To achieve a compromise between sensitivity and precision and keeping in mind tests practicability, a sorption time of 16 hours (overnight) and a velocity of 300 rpm were selected as the optimal time and agitation rate for the enrichment of the priority pesticides into the PDMS-phase

3.3.2.2.3 Back extraction: desorption time, stirring rate and solvent

The influence of the time on the desorption of the pesticides from the PDMS stir bars was evaluated for a period of 15 min at 200 rpm and 500 rpm and also for a period of 30 min at 200 rpm. An increase in the stirring rate did not improve analytes desorption probably due a certain instability of the stir rods at such velocity. Except for mirex, no fluctuations in peak areas were observed when the time increased from 15 minutes to 30 minutes and therefore, a period of 15 min was selected as the optimal desorption time. Relatively to solvents, ethyl acetate and a mixture of *n*-hexane and acetone (9:1) were the solvents tested for pesticides back extraction (desorption). High peak areas were obtained with *n*-hexane:acetone (9:1). γ-Chlordane was an exception showing better results when ethyl acetate was used. Acetone was added to hexane to decrease the percentage of swelling of PDMS-phase and to increase the solubility of some pesticides [40]. Chlorinated solvents such as methylene chloride were not considered because they might damage the stir bars PDMS-phase [41].

3.3.3 Sample amount and solvent volume optimization

Once the ultrasonic settings and the SBSE parameters have been optimized the effect of sample amount and solvent volume on the extraction of the priority pesticides was evaluated. All the extractions were done with acetonitrile. No investigation were made with other solvents since acetonitrile

has been successfully applied to standard multi-residue methods developed for analysis of pesticides in plants and food samples [42].

Lower recoveries were obtained for all pesticides at a sample/volume ratio of 10g:20mL, as illustrated in Fig. 3.3 by aldrin, hexachlorobenzene and mirex. When 10 g of sample were used recoveries increased as the volume of acetonitrile decreased. For 15 g some variations were observed as illustrated in Fig. 3.3. Except for β-HCH, better results were found at a sample/volume ratio of 10g:10 mL. An increment of almost 60% was observed on mirex extractability when the extraction was carried at such ratio. Good conditions were accepted when recoveries were in the range 70-120% for the majority of the pesticides [18].

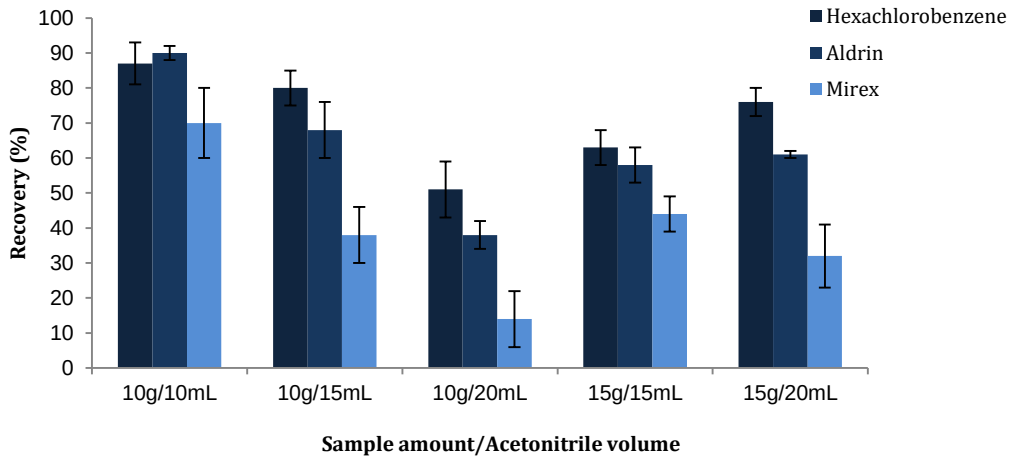


Fig. 3.3 – Influence of sample amount and volume of acetonitrile on the extraction of the priority pesticides from marine sediments. Results are presented as the mean ± standard deviation.

3.3.4 Method validation

The developed method was validated relatively to linearity, limit of detection (LOD) and quantification (LOQ), precision and accuracy using optimal conditions for extraction (3.2.4.1.3.) and analytes enrichment (3.2.4.2.2.).

3.3.4.1 Linearity and limits of detection and quantification

Linearity was assessed using matrix-matched calibration solutions over a range between the method quantification limit (MQL) and 70 ngg^{-1}. Six concentration levels were studied using triphenylmethylchloride as internal standard (IS). Good linearity of the response was found at the selected concentration range. The correlation coefficient (r^2) was >0.99 for all analytes (Table 3.2) in accordance with the quality control reference guidelines for pesticides analysis [18]. Limits of detection (LODs) and limits of quantification (LOQs) were established from spiked blank extracts employing a signal-to-noise (S/N) of 3 and 10, respectively. LODs ranged from 0.02 ngg^{-1} to 1.6 ngg^{-1} and the LOQs from 0.09 ngg^{-1} to 3.5 ngg^{-1}. Results are comparable to detection limits that have been found by other researcher groups who have used UAE as well as other sample preparation techniques [43-49].

Table 3.2 – Linearity (correlation coefficient, r^2), limits of detection (LOD), limits of quantification (LOQ) and respective method detection limits (MDL) and quantification limits (MQL).

Compound Name	Correlation coefficient (r^2)	LOD (ngg^{-1})	LOQ (ngg^{-1})	MDL (ngg^{-1})	MQL (ngg^{-1})
Hexachloro-1,3-butadiene	0.9972	0.02	0.09	0.27	0.82
Trifluralin	0.9981	0.16	0.31	0.41	1.2
α-HCH	0.9977	0.16	0.60	0.24	0.71
Hexachlorobenzene	0.9993	0.19	0.36	0.55	1.8
β-HCH	0.9992	0.19	0.36	0.57	1.7
γ-HCH (Lindane)	0.9990	0.16	0.60	0.48	1.4
Quintozene	0.9983	0.95	2.1	0.89	2.7
Terbuthylazine[a]	0.9999	0.84	1.6	4.6	14
δ-HCH	0.9992	0.16	0.45	1.0	3.1
Heptachlor	0.9982	0.16	0.30	0.51	1.5
Alachlor	0.9993	0.16	0.30	1.0	2.9
Aldrin	0.9989	0.16	0.30	0.62	1.9
Chlorpyrifos	0.9987	0.32	0.61	0.42	1.2
Isodrin	0.9982	0.19	0.73	0.93	2.8
Heptachlor epoxide	0.9998	0.16	0.30	0.48	1.4
Chlorfenvinphos[a]	0.9977	1.6	3.5	4.4	13
γ-Chlordane	0.9996	0.08	0.15	0.36	1.1
α-Endosulfan	0.9963	0.32	1.50	0.61	1.8
Dieldrin	0.9979	0.16	0.30	0.43	1.3
p,p′-DDE	0.9996	0.32	0.60	0.34	1.0
Endrin	0.9999	0.30	0.90	0.82	2.5
β-Endosulfan	0.9965	0.32	1.5	0.56	1.7
p,p′-DDD	0.9972	0.16	0.60	0.43	1.3
Endosulfan sulphate[a]	0.9997	0.32	1.2	2.2	6.6
p,p′-DDT	0.9995	0.30	0.9	0.31	0.9
Bromopropylate	0.9988	0.31	0.94	0.80	2.4
p,p′-Methoxychlor	0.9991	0.30	0.45	1.0	2.9
Mirex	0.9956	0.16	1.1	0.91	2.7

[a]MDL was calculated with a spiked samples at 10 ngg^{-1}.

To take into account the overall analytical procedure, method detection limits (MDLs) and method quantification limits (MQLs) were also evaluated. Based on relevant references, MDLs were calculated by: $MDL = st_{(0.99,\ n-1)}$, where s is the standard deviation of four replicates and $t_{(0.99,\ n-1)}$, is the t-distribution value taken at a confidence level of 99% and n the degrees of freedom [12, 50-53]. In this study, MDLs were statistically estimated from the analysis of four replicated sediments samples spiked at 2.5 ngg^{-1}. Method quantification limits (MQLs) were calculated by: $MQL = 3xMDL$ [50, 52]. Since the sampling area comprises mainly two type of marine sediments, namely sandy and sandy-loam (muddy) material, replications where carried out using those types sediments. A total of four replicates at 2.5 ngg^{-1} were performed using two spiked replicates of each type of sediment. In this way the calculation of the MDLs and MQLs took into account the heterogeneity and complexity of the different analyzed sediments samples. Not so often reported, MDLs are always higher than LODs and tend to describe the precision of quantification for each compound in the method, while LODs describe the certainty of the instrument detection [12]. As can been seen from Table 3.2, MDLs ranged from 0.3 to 4.4 ngg^{-1} while MQLs from 0.8 to 14 ngg^{-1}. Higher MDLs values were found by M. Lyytikäien et al. [54]. The authors extracted five classes of pesticides by a modified standard procedure of the USEPA method 3550 (sonication extraction for low concentrations of organic compounds). Compounds were further and analyzed by GC-ECD (electron capture detector) and GC-NPD (nitrogen-phosphorous detector). Higher values were also found by K. L. Smalling et at. [12] in the analysis of a variety of legacy pesticides extracted by microwave-assisted extraction. Except for chlorfenvinphos, higher MDLs, in the range of 2 to 4 ngg^{-1}, were found by K. Kawata et al. [53] in the analysis of several class of pesticides extracted by UAE and pre-concentrated by solid phase extraction (SPE). A MLD of 0.45 ngg^{-1} and a MQL of 1.4 ngg^{-1} were found by H. Li et at. [55] for chlorpyrifos in

the analysis of organophosphate and pyrethroid insecticides by ultrasound-assisted microwave extraction followed by GC-MS.

3.3.4.2 Accuracy and precision

Accuracy was evaluated by means of pesticides recoveries at a low level of spiking of 2.5 ngg^{-1} and at a high level of 70 ngg^{-1}. Assays where carried out using the two types of marine sediments samples (sandy and muddy materials) as reported in Table 3.3. Recoveries in the range of 70 - 111% were found for the majority of the pesticides in accordance with the established criteria of pesticides quality control guidelines (70 – 120%)[18]. The metabolite heptachlor epoxide and terbuthylazine were an exception. Lower recoveries were found for terbuthylazine in sandy sediments. Similar recoveries in the range of 63% to 119%, were found by F. J. Camino-Sanchés [34] in the analysis of POPs in marine sediments by pressurized liquid extraction followed by SBSE and GC-MS/MS. Identical values were obtained by K. L. Smalling et at. [12] who developed a multi-residue analytical method for the analysis of a variety of legacy pesticides by GC-μEC, with GC-MS confirmation, using microwave-assisted extraction as sample preparation technique. Chlorpyrifos also showed recoveries in the same range as the one obtained by H. Li [55] in the analysis of organophosphate insecticides by GC-MS using ultrasound-assisted microwave as extraction technique. Comparable results were found by K. Kawata [53] for alachlor, chlorfenvinphos, chlorpyrifos, quintozene and trifluralin, which were doubled extracted by UAE and isolated by SPE. The same type of extraction and pre-concentration technique was used by Min-Su Kim et al. [47] in the analysis of organochloride pesticides by GC-MS. To improve efficiency extractions were carried out in duplicate [47]. Recoveries between 73% to 106% are in good agreement with the values found in this work. O.P. Jiménez et al. [49] used UAE associated to traditional sample clean-up (copper addition followed by elution through silica gel and alumina) and obtained pesticides recoveries using three extraction cycles in the range

of 40% to 93%. Methoxychlor, *p,p'*-DDD, *p,p'*-DDT and endosulfan sulphate presented lower values than the ones found herein.

Table 3.3 – Recoveries of priority pesticides at low and high level of spiking into different types of marine sediments samples (sandy and muddy materials). Values are reported as mean ± standard deviation.

	Sandy			Muddy		
	2.5 ngg^{-1}	70 ngg^{-1}		2.5 ngg^{-1}	70 ngg^{-1}	
Compound Name	**Recovery (%)**	**Recovery (%)**	**Mean RSD (%)**	**Recovery (%)**	**Recovery (%)**	**Mean RSD (%)**
Hexachloro-1,3-butadiene	75 ± 1	101 ± 12	12	79 ± 1	103 ± 4	4
Trifluralin	90 ± 1	74 ± 8	8	92 ± 6	89 ± 3	3
α-HCH	108 ± 2	83 ± 11	11	107 ± 3	105 ± 11	12
Hexachlorobenzene	87 ± 3	87 ± 2	2	93 ± 2	92 ± 10	11
β-HCH	83 ± 9	71 ± 5	13	85 ± 10	98 ± 8	9
γ-HCH (Lindane)	88 ± 5	70 ± 3	3	83 ± 1	109 ± 6	6
Quintozene	83 ± 1	73 ± 2	2	92 ± 10	83 ± 12	14
Terbuthylazine[a]	59 ± 3	61 ± 9	6	80 ± 12	103 ± 8	10
δ-HCH	90 ± 1	73 ± 3	5	92 ± 1	91 ± 2	2
Heptachlor	90 ± 1	102 ± 4	4	92 ± 7	99 ± 1	1
Alachlor	75 ± 2	84 ± 10	10	90 ± 2	102 ± 3	3
Aldrin	92 ± 7	90 ± 3	5	88 ± 6	93 ± 5	6
Chlorpyrifos	104 ± 5	80 ± 2	4	105 ± 3	100 ± 5	4
Isodrin	77 ± 2	82 ± 2	5	69 ± 4	88 ± 2	4
Heptachlor epoxide	60 ± 1	50 ± 2	2	66 ± 3	66 ± 9	7
Chlorfenvinphos[a]	81 ± 2	111 ± 6	3	105 ± 5	101 ± 13	9
γ-Chlordane	95 ± 5	70 ± 2	4	97 ± 1	78 ± 2	2
α-Endosulfan	98 ± 1	81 ± 2	2	102 ± 8	104 ± 5	2
Dieldrin	80 ± 1	85 ± 1	1	74 ± 2	106 ± 9	6
p,p′-DDE	84 ± 3	99 ± 4	4	80 ± 2	101 ± 9	6
Endrin	75 ± 1	81 ± 1	2	85 ± 2	103 ± 1	2
β-Endosulfan	93 ± 8	93 ± 7	6	93 ± 1	103 ± 6	3
p,p′-DDD	90 ± 1	92 ± 3	2	90 ± 6	101 ± 4	5
Endosulfan sulphate[a]	103 ± 4	87 ± 11	8	106 ± 4	109 ± 3	3
p,p′-DDT	80 ± 3	84 ±2	4	83 ± 1	88 ± 8	1
Bromopropylate	83 ± 1	75 ± 4	2	90 ± 10	101 ± 6	8
p,p′-Methoxychlor	81 ± 1	74 ± 6	5	96 ± 1	87 ± 1	1
Mirex	90 ± 10	71 ± 8	11	81 ± 3	70 ± 2	3

[a]Recovery was calculated at a low level of 10 ngg^{-1} (low spike level).

Precision, expressed as relative standard deviation (RSD), was evaluated for each type of marine sediment sample. An average value of the two spiking levels was calculated and reported in Table 3.3. The obtained RSD are well below 20% being in good conformity with the quality control reference guidelines [18] as well as with the relevant published data [12, 34, 47, 48, 53-55]. To confirm the method performance and to evaluate the extraction from aged compounds a marine sediment standard reference material (SRM 1941b, organics in marine sediments) was analyzed. The obtained values are within the uncertainty range (Table 3.4).

Table 3.4 – Results of the Standard Reference Material (SRM 1941b) analysis.

Priority Pesticide	Concentration (ngg^{-1}, dry weight)		
	Certified	Obtained[a]	Recovery[a] (%)
Hexachlorobenzene	5.83 ± 0.38	5.65 ± 0.14	97 ± 2
γ-Chlordane	0.57 ± 0.09	0.54 ± 0.09	95 ± 16
p,p'-DDE	3.22 ± 0.28	3.29 ± 0.23	102 ± 7
p,p '-DDD	4.66 ± 0.46	4.55 ± 0.07	98 ± 1
p,p '-DDT	1.12 ± 0.42	0.90 ± 0.17	81 ± 15

[a] mean ± standard deviation (n = 2).

Results obtained and validated in this work proved that the application of UAE at high temperatures can be a simple and efficient alternative to other sample preparation techniques that use much more expensive equipment. Moreover, the application of UAE at high temperatures reduces the number of extraction cycles. This is an advantage over the normal procedure of UAE where extractions are always carried out with 2 to 5 cycles [46, 47, 53, 56-58]. Moreover, a total time of almost 17h may appear to be a time-consuming step, however, when compared with the traditional procedures (extraction plus sample clean-up) the method developed herein is faster [59].

3.4 Field application

After optimisation and validation the method was applied to sediment samples collected from 8 different places along the Barrosa branch of Óbidos

Lagoon (Annex B, Fig. B.1). The concentrations (ngg^{-1}) of the compounds in the sediment samples are presented in Table 3.5. Method performance was checked by application of the internal quality control described in Section 3.2.6. No interfering peaks were found in blanks, the RDS of duplicate samples were <20% and the recovery of spiking samples from site 4 and 8 were between 70 – 120%.

Table 3.5 – Concentration of priority pesticides (ngg^{-1}, dry weight) in samples collected in the 8 different sites along the Barrosa branch of Óbidos Lagoon.

	Concentration (ngg^{-1}) in the 8 sampling sites							
Priority Pesticide	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Hexachlorobutadiene	*	*	*	*	11.1	8.5	5.4	*
Trifluralin	*	*	*	*	4.5	4.1	3.4	*
α-HCH	*	*	*	*	9.7	6.2	5.5	*
Hexachlorobenzene	*	*	*	*	14.3	10.8	10.3	*
β-HCH	*	*	*	*	*	*	*	*
γ-HCH	*	*	*	*	13.6	*	*	*
Quintozene	*	*	*	*	7.5	5.8	5.2	*
Terbuthylazine	*	*	*	*	*	*	*	*
δ-HCH	*	*	*	*	*	8.1	*	*
Heptachlor	*	*	*	*	11.4	10.4	6.8	*
Alachlor	*	*	*	*	*	*	*	*
Aldrin	*	*	*	*	6.5	4.2	5.3	*
Chlorpyrifos	*	*	3.3	2.2	14.3	11.6	11.3	2.9
Isodrin	<MQL	<MQL	*	*	15.9	11.0	9.0	*
Heptachlor epoxide	*	*	*	<MDL	5.2	3.9	3.2	*
Chlorfenvinphos	*	*	*	*	35.5	41.1	33.8	*
γ-Chlordane	*	*	*	*	4.5	4.3	3.7	*
α-Endosulfan	*	*	*	*	11.6	9.5	5.8	*
Dieldrin	<MDL	<MDL	*	<MDL	4.3	2.9	2.2	*
p,p'-DDE	<MQL	<MQL	<MQL	<MQL	10.2	6.8	6.3	<MQL
Endrin	*	*	*	*	16.5	14.3	11.1	*
β-Endosulfan	*	*	*	*	8.7	6.8	4.0	*
p,p '-DDD	*	*	*	*	7.3	4.3	3.8	*
Endosulfan sulfate	*	*	*	*	7.8	*	*	*
p,p '-DDT	*	*	*	*	7.4	5.4	4.8	*
Methoxychlor	*	*	*	*	12.2	6.9	7.9	*
Mirex	*	*	*	*	5.7	5.4	6.0	*

* not detected; MDL - method detection limit; MQL – method quantification limit.

Most of the priority pesticides analysed were found in the muddy sediments from sites 5, 6 and 7. Among them, chlorfenvinphos presented the highest concentration, presumably to its broad application as animal ectoparasiticide in livestock and wineries in the area [60]. Concentrations of *p,p'*-DDT registered in the current work are similar to the values reported in sediments collected in 2001 from the same area [61]. Otherwise, an enhancement of one order of magnitude was found for dieldrin. It should not be negligible possible contributions of small tributaries in the rainy periods [61, 62].

3.5 Conclusions

Multi-frequency ultrasonic baths are a promising tool in the analytical field. Several parameters like frequency, power intensity, signal modulation and temperature can be evaluated and optimized. To reach maximum extraction a compromise between the intensity and the frequency must be met. In this study it was explored the combination of ultrasounds irradiation with high temperatures. Extractions at low frequencies and low intensities with temperatures near the boiling point of acetonitrile (82 ^{o}C) showed a positive effect on the extractability of priority pesticides, especially for those that tend to bind tightly to soil and sediments. Moreover, the application of ultrasounds at high temperatures reduced the number of extraction cycles. In the sweep mode of operation, the distribution of the ultrasounds across the tank was more effective at lower frequencies. Except for heptachlor epoxide and terbuthylazine, good recoveries, at high levels of precision, were achieved in accordance with quality control guidelines. The optimized ultrasonic-assisted extraction (UAE), in association with stir bar sorptive extraction (SBSE) technique used as sample clean-up and preconcentration, showed to be a simple and effective sample preparation procedure for the screening of priority pesticides in different types of marine sediments by GC-MS/SIM, being a good

alternative to other sample preparation procedures that use much more expensive equipment.

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4

Screening of Priority Pesticides in *Ulva* sp. Seaweeds by Selective Pressurized Solvent Extraction before Gas Chromatography with Electron Capture Detector Analysis

Highlights

- Pesticides extraction and sample clean-up were carried out in one single step.
- Good performance for a high number of pesticides was achieved by SPLE-GC-ECD.
- Application of SPLE methodology reduced significantly the total time of analysis.
- *Ulva* sp. seaweeds accumulated pesticides after long periods of storm events.
- Seaweeds showed to be promising analytical matrix for pesticides runoff monitoring.

❖ M. I. Pinto, C. Micaelo, C. Vale, G. Sontag, J. P. Noronha, Screening of priority pesticides in *Ulva* sp. seaweeds by selective pressurized solvent extraction before gas chromatography with electron capture detector analysis, **Arch. Environ. Cont. Tox.**, 67 (2014), 547-556 (DOI 10.1007/s00244-014-0038-2).

Abstract

This work reports a fast and reliable analytical method for the screening of priority pesticides (PPs) in *Ulva* sp. seaweeds by gas chromatography with electron capture detection. Extraction and sample clean-up were performed in one single step by selective pressurized liquid extraction (SPLE). Several parameters affecting SPLE performance were optimized. Method performance was compared with standard Soxhlet extraction. Significant decrease of the time of analysis with better recoveries for a greater number of PPs was achieved by SPLE. Average recoveries ranged from 71 to 103 % with RSD < 10 %. Field application showed the presence of PPs in the range of 3 - 11 ngg^{-1} in seaweeds collected in a coastal lagoon after a long period of heavy rains. These results suggest that *Ulva* sp. seaweeds tend to accumulate PPs and have the potential to be used as early alert signals of aquatic pollution especially after rains and storm events.

Keywords: Organohalogenated compounds; Macroalgae; Sample clean-up; Accelerated solvent extraction; Coastal lagoons; Runoff.

4.1. Introduction

Priority pesticides (PPs), listed under Annex X of Water Framework Directive, are a group of banned and toxic substances that are known by their persistency in the aquatic environment [1, 2]. Most of them are extremely hydrophobic and once they enter the aquatic ecosystem they tend to be adsorbed or absorbed by sediments and incorporated into bioorganic matrices like marine macroalgae [3, 4]. In coastal lagoons, *Ulva* sp. seaweeds can growth massively as a consequence of the anthropogenic nutrient inputs [5, 6]. This type of marine macroalgae can be highly contaminated with hydrophobic pollutants and can work as a suitable analytical matrix for pollutants monitoring providing information on compounds availability and water quality deterioration [5-8]. It can also provide background data for possible bioremediation processes and ecological risks assessment.

To date the most common extraction technique applied to macroalgae PPs analysis has been soxhlet extraction followed by gel permeation or column chromatography as sample clean-up [3, 4]. Although valid and reliable, soxhlet extraction is a traditional procedure that uses large amounts of solvents. Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) was developed as green alternative to soxhlet [9-11]. Shorter extractions are carried out with much less volumes of solvents. Moreover, the possibility of in-cell sample clean-up increases the selectivity of the extractions and reduces significantly the total time of the analysis [10]. The main drawback is the initial cost of the PLE equipments. To the best of our knowledge, no studies have been done concerning the application of in-cell or selective pressurized liquid extraction (SPLE) to the analysis of PP in green seaweeds. Therefore, the aim of this study was to develop a rapid and selective PLE sample preparation method for the analysis of PPs in *Ulva* sp. seaweeds by gas chromatography with electron capture detector (GC-ECD). After validation the method was be applied as a screening tool for the analysis of PPs accumulation in wild *Ulva* sp. seaweeds.

4.2. Materials and methods

4.2.1. Equipment

A Dionex ASE 200 accelerated solvent extractor with stainless-steel cells of 33 mL (Sunnyvale, California, USA) was used to extract the PPs. Extracts were collected in vials of 60 mL. The GC-ECD analysis were carried out on a Hewlett-Packard 6890 Series Plus gas chromatographic system equipped with an Electron Capture Detector and a Hewlett-Packard 7683 Series injector autosampler (Stevens Creek Boulevard, Santa Clara).

4.2.2. Reagents and standard solutions

A mixture of priority pesticides with aldrin, dieldrin, endrin, α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH, δ-HCH, *p,p'*-dichloro-diphenyldichloroethylene (*p,p'*-DDE), *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), *p,p'*-dichlorodiphenyltrichloroethane (*p,p'*-DDT), α-endosulfan, heptachlor, heptachlor epoxide and *p,p'*-methoxychlor was obtained from Supelco (EPA organochloride pesticide mix n.º46960-U, Bellefonte, PA, USA) at individually concentrations of 2000 μgmL^{-1} in *n*-hexane:toluene (1:1). γ-Chlordane (100 μgmL^{-1}) in *n*-hexane was also purchased from Supelco (Bellefonte, PA, USA). The standards pesticides bromopropylate (surrogate), chlorpyrifos, hexachlorobenzene (HCB), isodrin, mirex, quintozene, and trifluralin were purchased from Sigma-Aldrich (St. Louis, MS, USA) with a high purity level (97.0 - 99.9%). Acetone, *n*-hexane, toluene, ethyl acetate, cyclohexane and graphitized carbon black were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium sulphate anhydrous, Florisil (0.150 - 0.250 mm), aluminium oxide 90 active acidic (acidic alumina), aluminium oxide 90 active neutral (neutral alumina), aluminium oxide 90 active basic (basic alumina) and silica gel 60 were obtained from Merck KGaA, (Darmstadt, Germany). Magnesium sulphate anhydrous was purchased from Scharlau, Chemie S.A. (Sentimental, Spain) and powder molecular sieves type 4A from Fluka (St. Louis, MS, USA). The absorbing moisture polymer, under

the trade name of Dionex, ASE Prep MAP, the cellulose filters and the calcinated diatomaceous earth (DE) were purchase from ThermoFisher Scientific (Sunnyvale, California). Cellulose extraction thimbles were obtained from Whatman, Schleicher & Schuell (Maidstone, England). The individually purchased pesticides were dissolved to 2000 μgmL^{-1} in *n*-hexane or *n*-hexane:acetone (2:1) depending on their solubility in organic solvents. A mixture of all the pesticides at a concentration of 10 $\mu g\ mL^{-1}$ was further prepared by appropriate dilution of the stock standard solutions in *n*-hexane:acetone (9:1). The internal standard (IS) *p,p'*-dibromodiphenyl was obtained from Sigma-Aldrich (St. Louis, MS, USA) and prepared at a concentration of 2000 μgmL^{-1} in *n*-hexane:acetone (2:1). Internal standard stock solution was diluted to 5 μgmL^{-1} in *n*-hexane:acetone (9:1). The pesticide *p,p'*-DDT used in quality control tests was obtained from Sigma-Aldrich (St. Louis, MS, USA). A solution of *p,p'*-DDT was prepared in toluene at a concentration of 100 μgmL^{-1} and diluted to 1 μgmL^{-1} in *n*-hexane. All the solutions were protected from light and kept at 4 ^{o}C.

4.2.3. Sampling

Sampling was performed in the upper branch of Óbidos Lagoon, which receives the discharge of an intense agriculture area [12, 13]. Samples were collected in winter (February 2013) after a long period of heavy rains and in summer (August 2013). After collection, *Ulva* sp. samples were triturated, homogenized, and stored at -20 °C until analysis (EN 15662 2007) [14]. Method validation was performed with samples collected from an aquaculture earthen pond.

4.2.4. Sample preparation

4.2.4.1 SPLE: Optimization

Optimization of the extraction and simultaneous in-cell sample clean-up was performed according to the plan listed in Table 4.1. Thawed samples

were weighted and spiked with a mixture of PPs at a final concentration of 100 ngg^{-1}. After spiking, samples were allowed to equilibrated for 1 hour and further mixed in a mortar with the drying agent or desiccant and 1 g of diatomaceous earth (DE) as dispersing agent. The cell was filled with the mixture as shown in Table 4.1. DE was further added to fill the remaining empty space. The influence of the temperature, number of cycles, preheating time and static time was evaluated as well as the extraction efficiency of the different types of organic solvents. Six types of sorbents were tested as listed in Table 4.1. Moisture retention was also evaluated for five desiccants. Dionex ASE Prep MAP absorbing polymer was tested in accordance with supplier information [15].

4.2.4.2 SPLE: Optimal conditions

Extractions and simultaneous sample clean-up were performed with 5 ± 0.01 g of *Ulva* sp. sample and 2.5 ± 0.01 g of Florisil. Thawed samples were weighted and mixed in a mortar with 10 ± 0.1 g of sodium sulfate anhydrous and 1 g of DE. Samples were transferred into the cell and allowed to dry for 1hour at room temperature. The cells were loaded as shown in Table 4.1. To increase the extraction of trifluralin, 0.5 mL of ethyl acetate were added to the cell. Extractions were carried out with *n*-hexane using one cycle at 120 ^{o}C, 1500 psi, a static time of 5 min without a preheating time, a flush volume of 60% and a nitrogen purge of 150 seconds. The extracts were collected in vials of 60 mL. In-vial moisture removal was carried out with sodium sulfate to insure that there was no water present in the final extracts. To finish, the extracts were concentrated by rotary evaporation to approximately 2 mL, then transferred to a graduated tube, spiked with the internal standard (*p,p'*-dibromodiphenyl) and taken for evaporation under a gentle stream of nitrogen to a final volume of 0.5 mL.

4.2.5. Soxhlet extraction

Thawed samples were weighted (5 ± 0.01 g) and spiked with a mixture of PPs at a final concentration of 100 ngg^{-1}. After spiking, samples were allowed to equilibrated for 1 hour and further mixed in a mortar with the 15 g of sodium sulfate anhydrous and 1 g of DE. The mixture was transferred into cellulose extraction thimbles and the extraction was carried out with *n*-hexane for 17 hours. The extracts were purified in a Florisil column (1% H_2O) and eluted with *n*-hexane and *n*-hexane/dichloromethane (7:3) [16-18].

Table 4.1 - Tested parameters and experimental conditions used in the SPLE optimization. Optimal conditions were used in method validation and field application.

Cell filling	Parameters and experimental conditions	Optimization	Optimal conditions
	**Temperature** *n*-hexane, 5 min, 1 cycle, 2.5 g Florisil, 5 g sample, 15 g sodium sulfate	80 ºC, 100 ºC, 120 ºC, 150 ºC	120 ºC
	Number of Cycles *n*-hexane, 120 ºC, 5 min, 2.5 g Florisil, 5 g sample, 15 g sodium sulfate	1, 2 and 3 cycles	1 cycle
	Preheating time *n*-hexane, 120 ºC, 5 min, 2.5 g Florisil, 5 g sample, 15 g sodium sulfate	0 and 5 min	0 min
	Static time *n*-hexane, 120 ºC, 1 cycle, 2.5 g Florisil, 5 g sample, 15 g sodium sulfate	5, 10 and 20 min	5 min
	Solvent 120 ºC, 5 min, 1 cycle, 2.5 g Florisil, 5 g sample, 15 g sodium sulfate	*n*-Hexane *n*-Hexane:acetone (9:1) *n*-Hexane: ethyl acetate (9:1) *n*-Hexane: toluene (2:1) Cyclohexane: ethyl acetate (2:1) Ethyl acetate	*n*-Hexane
	Sorbent for in-cell clean-up *n*-hexane, 120 ºC, 5 min, 1 cycle, 5 g sample, 15 g sodium sulfate, 2.5 g sorbent (except, GCB 1.0 g). Florisil was tested with 2.5 g, 5 g and 1 g.	Florisil, alumina (acidic, neutral and basic), GCB	Florisil (2.5 g)
	Drying agent or desiccant *n*-hexane, 120 ºC, 5 min, 1 cycle, 2.5 Florisil, 5 g sample and desiccant.	Sodium sulfate anhydrous (15 and 10 g), magnesium sulfate anhydrous (10 g), molecular sieves (5 g), Dionex ASE Prep MAP (2 g absorbing polymer) and DE	Sodium sulfate anhydrous (10 g)

Note: All tests were carried out at 1500 psi, 60 % flush, 150 s purge.

4.2.6. GC-ECD analysis

The GC column used to separate the compounds was a DB-5 silica capillary column (30 m x 0.25 mm i.d.; d_f: 0.25 µm, Agilent-J&W Scientific). Helium at 1 $mLmin^{-1}$ was used as carrier gas and a mixture of methane:argon (5:95) was used as the make-up gas at a linear velocity of 60 $mLmin^{-1}$. The electron capture detector was operating at 320 ºC. GC injection parameters were as follows: 2 µL of solution in a splitless mode; injector temperature 280 ºC. GC temperatures: 80 ºC isothermal of 1 min, 25 ºC min^{-1} to 140 ºC, 2 ºC min^{-1} to 190 ºC isothermal 2.00 min, 3 ºC min^{-1} to 215 ºC isothermal 2.00 min, 16 ºC min^{-1} to 280 ºC isothermal 4.60 min. Data were acquired and processed using the Agilent ChemStation software. Identification of priority pesticides was done by comparison of the retention time with the respective standards. Quantification was performed by multi-level calibration. An evaluation of the *4,4´*-DDT breakdown was used to check the liner performance [19].

4.2.7. Internal quality control

Internal quality control was made by running blanks, spiked samples (recovery) and samples in triplicate (precision and assessment of sample heterogeneity and matrix effects). Acceptance criteria were established in accordance with the quality control guidelines: (1) blanks with no interferences; (2) precision ≤20%; (3) recoveries in the range of 70% to 120% and (4) *p,p'*-DDT breakdown ≤20% [19, 20].

4.3. Results and discussion

To avoid a massive presentation and for a better visualization of the trends, the results of the optimization process were illustrated only for some representative PPs. The optimization was performed for all of the studied pesticides, and all of the obtained data can be found in Annex B (Fig. B.2 to B. 9).

4.3.1. Extraction and sample clean-up optimization

4.3.1.1 Selection of temperature, number of cycles, preheating and extraction time

Temperature was the first variable to be optimized. Results have shown that for the majority of the 21 studied compounds peak areas enhanced as temperature increased from 80 ºC to 120 ºC as shown in Fig. 4.1 for some representatives PPs. Aldrin and isodrin were an exception with higher responses at 100 ºC (Fig. 4.1a and Fig. B.2). At 150 ºC all peak areas decreased possibly as a consequence of pesticides degradation [21]. A decrease in peak areas was also noticed when extractions were carried out with more than one cycle as shown in Fig. 4.1b. In each new cycle, fresh solvent is in contact with the sample. In most of the cases, this improves the partition of the remaining analytes into the fresh solvent increasing the effectiveness of the extractions [22]. However, as referred by G. Ottonello et al. [22] an increase in the number of cycles is not a guarantee of higher recoveries. This behavior was notice also by Lorenzo et at. [23] in the analysis of azamethiphos, three avermectins, two carbamates and two benzolurea pesticides in seaweeds by SPLE before to liquid chromatography-mass spectrometry (LC-MS). The influence of the matrix under investigation and the extraction of interfering substances are pointed out as potential causes of such behavior [21, 22, 24]. In current work, the number of interfering substances did not increased with multiple cycles extraction. It is possible that the flush step had some influence on the efficacy of the PPs extraction. In the flush step, the cell is rinsed with a percentage of fresh solvent to drag the target analytes to the collection vial. When more than one cycle is specified, the total selected flush volume is divided among the cycles. Possibly, the fraction of solvent used to rinse the sample in each of the 2 a 3 cycles was not enough to transfer the remaining extracting solvent to the collection vial.

Another important parameter that was evaluated was the preheating time before the addition of solvent to the cell. An increase of 5 min was tested. The preheating time is the length of time the cell is preheated before the addition of solvent. As shown in Fig. 4.1c, peak areas decreased for aldrin and *p,p'*-DDT when preheating time increased. The same behavior was found for heptachlor, isodrin, *p,p'*-DDE, *p,p ´*-DDD and mirex. Contrary to the number of cycles, an increase on the number of interfering substances was registered especially at retention time of trifluralin raising the respective standard deviations (Annex B, Fig. B.3). Relatively to the static time, higher peak areas were found at 20 min for majority of the PP as shown in Fig. 4.1d for some representative pesticides. The isomers, β-HCH and γ-HCH were an exception with better results after 10 min of contact between sample and solvent. Heptachlor, aldrin, isodrin, heptachlor epoxide, *p,p'*-DDE, *p,p´*-DDD, *p,p'*-DDT, *p,p'*-methoxychlor and mirex were also an exception with high peak areas when static time was set as 5 min. The static time is the amount of time the solvent is in contact with the sample at the selected temperature and pressure. Although 20 min could be the best option, it was found out that the number of co-eluted substances increased as the static time increased. Therefore, 5 min were selected as the most favorable time for the extraction of the 21 PPs together with a temperature of 120 ^{o}C using one cycle of extraction without a preheating time. Other parameters like pressure, nitrogen purge and flush volume were programmed as 1500 psi, 150 s and 60%, respectively. Those parameters were set in accordance with published data [25, 26].

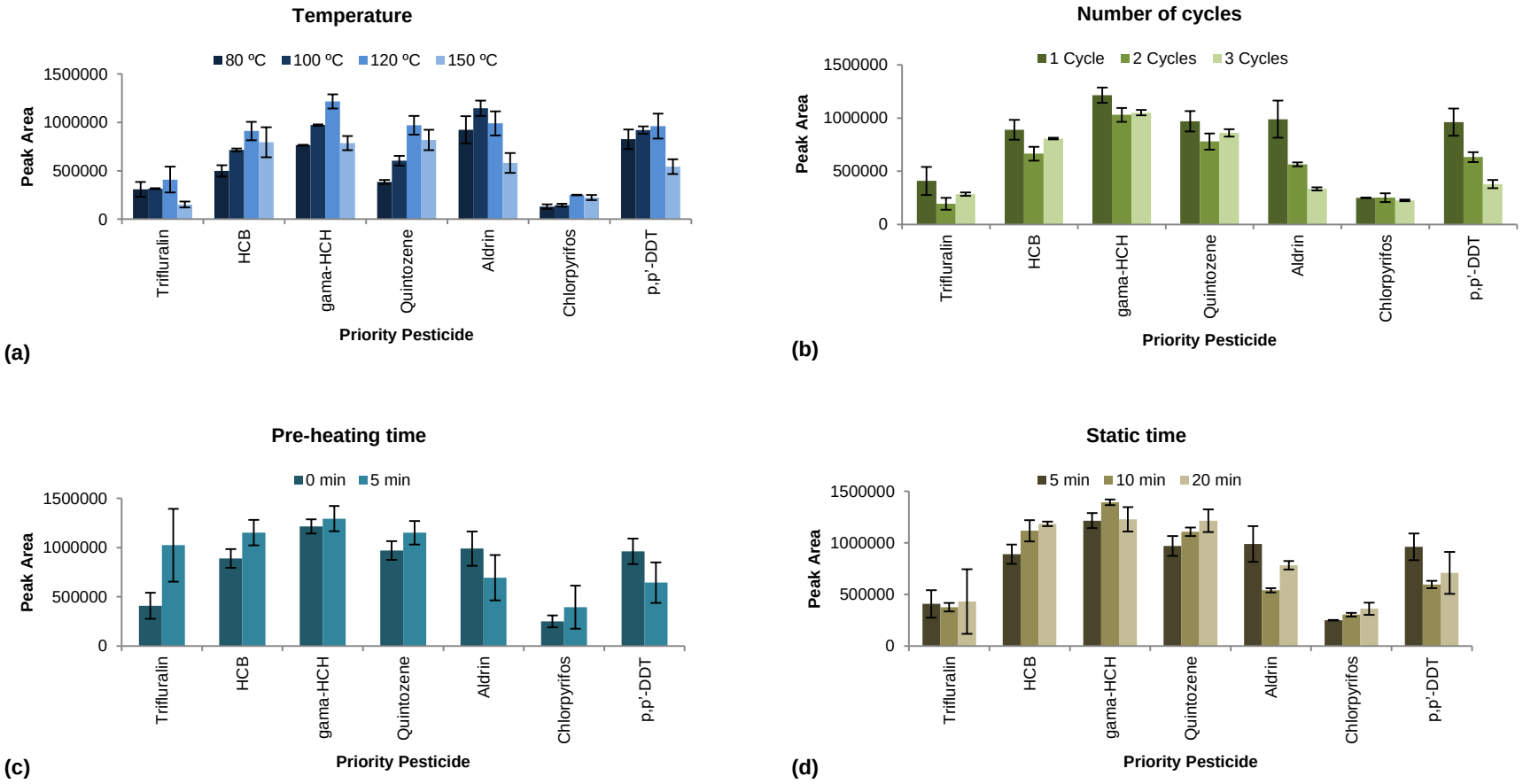

Fig. 4.1 - Influence of PLE operational parameters on extraction of some representative PPs: (**a**) Temperature, (**b**) number of cycles (**c**) preheating time and (**d**) static time. Results are presented as mean ± standard deviation (n = 2). Experimental conditions are summarized in Table 4.1. HCB - hexachlorobenzene.

4.3.1.2 Selection the extraction solvent

To extract the PPs from *Ulva* sp. samples different solvents and/or mix of solvents with different polarities were tested. Results are presented in Fig. 4.2. To avoid a massive presentation of all the studied compounds only some representative PPs were graphically illustrated. As can be seen from Fig. 4.2, extractions of the target compounds with ethyl acetate showed lower peak areas, except for trifluralin probably due to its high solubility in that solvent [27]. *n*-Hexane:acetone (9:1) presented higher values than the other solvent mixtures. For the majority of the PPs *n*-hexane showed better results. Exceptions were found for HCB, quintozene, chlorpyrifos and bromopropylate. For those pesticides, *n*-hexane:acetone (9:1) would be a better option. However, as with static time, the presence of interfering substances increased with acetone addition. Therefore, *n*-hexane was selected as the optimal solvent for PPs extraction.

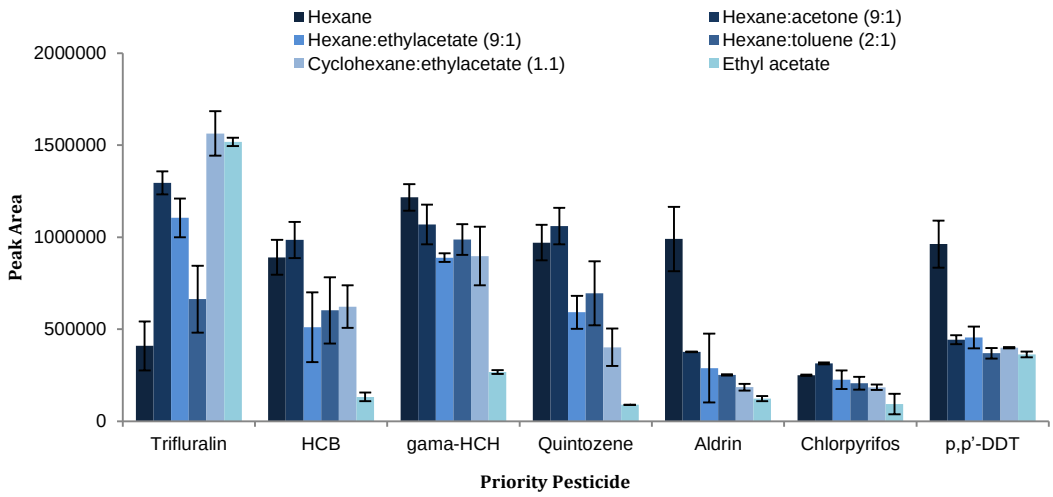

Fig. 4.2 - Influence of solvent on the extraction of some representative PPs. Results are presented as mean ± standard deviation (n = 2).

4.3.1.3 Efficacy of in-cell reagents: sample clean-up

Sample clean-up was evaluated for six different types of sorbents namely: Florisil, acidic alumina, neutral alumina, basic alumina, silica gel and graphitized carbon black (GCB). The sorbents were integrated directly into

the extraction cell. Higher peak areas were obtained with Florisil for all PPs as illustrated in Fig. 4.3a for some representative compounds. Among the different types of alumina sorbents, acidic alumina showed higher values followed by neutral and basic alumina. β-HCH, *p,p'*-DDE, mirex and *p,p'*-DDT were an exception with high values for neutral alumina (Fig. 4.3a, *p,p'*-DDT). Silica gel showed lower values than acidic, neutral and basic alumina, except for β-HCH, γ-HCH, δ-HCH, chlorpyrifos (Fig. 4.3a), α-endosulfan, and *p,p'*-DDE. Apart from Florisil, for those compounds silica gel would be a better option than basic alumina. The sorbent with the lowest values was GCB. Some pesticides like HCB, quintozene, chlorpyrifos and *p,p'*-methoxychlor were not extracted when GCB was used (Fig. 4.3a). GCB is an excellent sorbent of green pigments, however, planar pesticides like HCB and quintozene are retained by its structure [28, 29]. Chlorpyrifos and *p,p'*-methoxychlor are non-planar pesticides and their retention was probably a consequence of the amount of GCB used [29]. Recently, Florisil in conjunction with GCB was applied in the extraction of non-planar pesticides from seaweeds by SPLE [23, 30]. Three pyrethroids, a carbamate and two organophosphorus were analysed by García-Rodríguez et al. [30] by GC-MS/MS. The authors found out that a mixture of Florisil with GCB was a better cleanup option than neutral alumina. Pyrethroids and organophosphorus pesticides were also analysed by Lorenzo et al. [23] by LC-MS. In their work, silica combined with Florisil led to intensely colored extracts while GCB mixed with Florisil gave cleaner extracts. In the present work, pure Florisil was selected for in-cell sample clean-up and subsequent method validation. The application of GCB was not optimized since planar pesticides like HCB and quintozene were among the target analytes. From the results, and under the defined experimental conditions, it can be concluded that the efficacy and selectivity of the PLE in-cell clean-up, towards all the studied PP, depends on the type of sorbent used and decreases as follow: Florisil> acidic alumina> neutral alumina> silica gel> basic alumina> GCB.

4.3.1.4 Efficacy of in-cell reagents: sample drying

In this study *Ulva* sp. samples were dried by application of moisture adsorbents. Test were carried out with 5 different desiccants, namely: magnesium sulfate anhydrous, molecular sieves, sodium sulfate anhydrous, diatomaceous earth (DE) and a new moisture absorbing polymer under the trade name of Dionex, ASE Prep MAP. The lowest results were found for magnesium sulfate ($MgSO_4$) as shown in Fig. 4.3b for some representative PPs. These results are probably a consequence the high heat of hydration of $MgSO_4$ and its ability to retain some nonpolar compounds [31]. Molecular sieves are composed of crystalline zeolites (sodium and calcium aluminosilicates) and have a moderate heat capacity [31, 32]. As for $MgSO_4$, most of the PPs were retained except the planar pesticides HCB and quintozene (Fig. 4.3b). DE has a high water retention capacity, a low heat of hydration and a better sample consistency (sample dispersing) [31, 33]. Its application was more efficient in the extraction of some PP like chlorpyrifos, heptachlor epoxide and the HCH isomers (Fig. 4.3b, chlorpyrifos, γ-HCH). The ASE Prep MAP polymer showed lower values than Na_2SO_4 possibly as the result of the temperature used to extract the PPs [15]. The polymer has a high water retention capacity, however, higher amounts of the polymer must be used when extractions are carried out above 100 ^{o}C, which increases significantly the cost of the analysis. Sodium sulfate (Na_2SO_4) was the most efficient for the majority of the target analytes as illustrated in Fig. 4.3b for aldrin and *p,p'*-DDT. The amount of sodium sulfate (Na_2SO_4) was also optimized and better results were found at a concentration of 10 g of the salt per 5 g of *Ulva* sp. sample. Na_2SO_4 is a low cost desiccant and has a large water absorption capacity, however, as for $MgSO_4$ its drying capacity decreases when temperature increases [32]. The effect of salting out has been pointed out as the reason for a better efficacy of the inorganic salts when compared to other desiccants with higher water capacity like DE [33]. Although, the effect of salting out would benefit in the extraction of polar

compounds, little is known about the thermodynamic behavior of saturated solutions at high temperatures and high pressures.

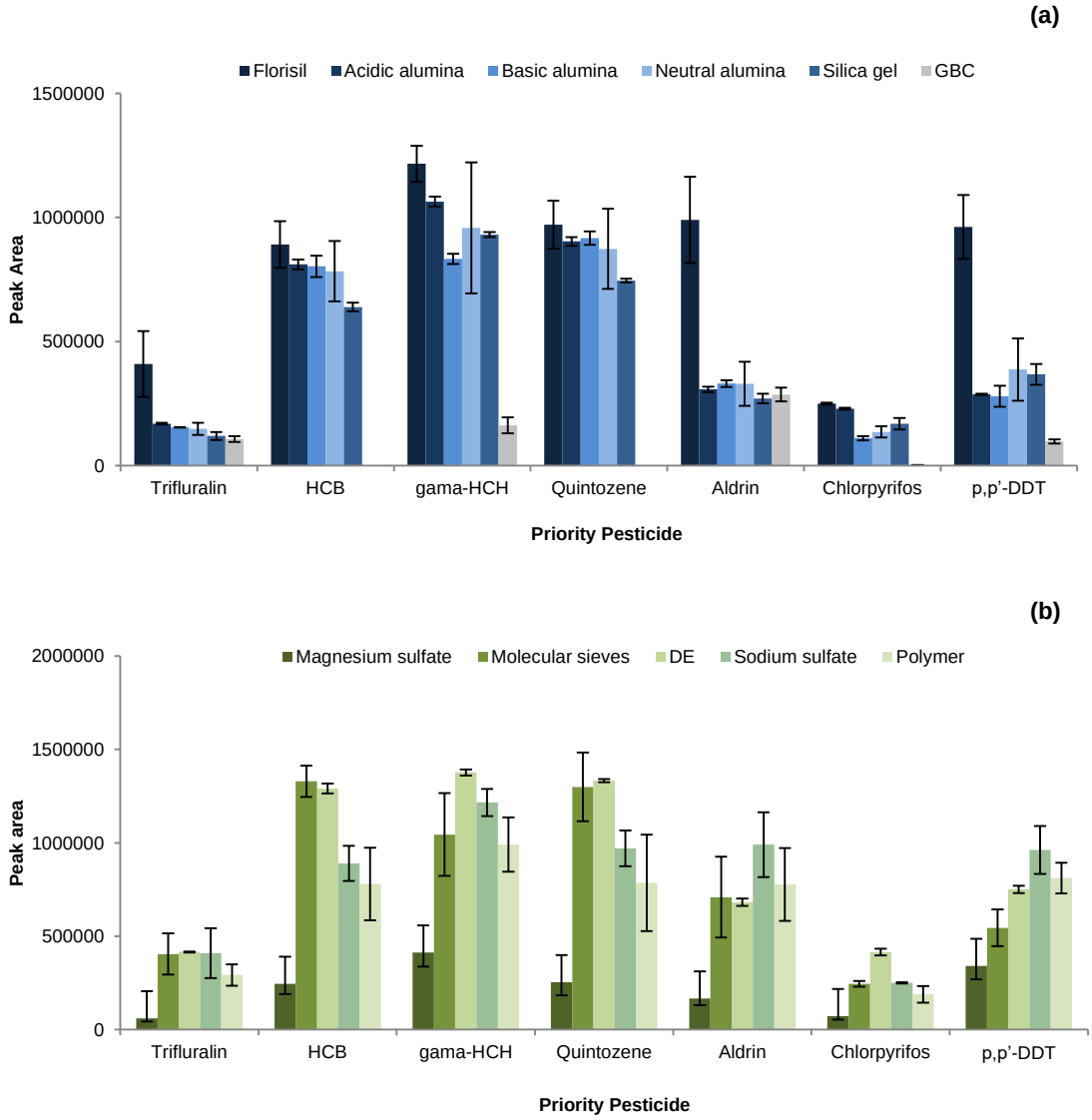

Fig. 4.3 - Effect of in-cell reagents on the selective of pesticides extraction: (**a**) Influence of adsorbent on sample clean-up; (**b**) Influence of desiccant on sample drying. Results are presented as mean ± standard deviation (n = 2). DE = diatomaceous earth, Polymer = Dionex ASE MAP Prep.

4.3.2. Method validation

4.3.2.1 Limits of detection and quantification

The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise (S/N) of 3 and 10, respectively

[20]. Results are presented in Table 4.2. LODs ranged from 0.2 to 3.2 ngg^{-1} and LOQs from 0.5 to 6.0 ngg^{-1} in a dry weight base. Lower LODs in the range of 0.1 ngg^{-1} were found by B. Pavoni et al. [4] and by L. Maroli et al. [3] in the analysis of DDT and its metabolites, HCB and α-HCC in seaweeds by GC-ECD. The authors used soxhlet extraction and column chromatography as sample clean-up. However, a high amount of freeze-dried sample was used which decreased the LODs. In the present work, samples were not lyophilizated to avoid the loss of the semi-volatile compounds. The founded values are below the 10 ngg^{-1}, which is the maximum residue level (MRL) of pesticides permitted in seaweeds for human consumption at the European Union [34]. In this way, the method can be applied not only for environmental purposes but also in the food analysis.

4.3.2.2 Matrix effect and linearity

Matrix effect was evaluated by comparison of peak areas of the target PPs dissolved in pure solvent (S) with those prepared in samples extract (A). The ratio of both measures (A/S × 100) is defined as the absolute matrix effect (ME) [35]. As shown in Table 2, %ME ranges from 91 - 109 % at a low (5 ngg^{-1}) and medium concentration level (25 ngg^{-1}). Since %ME was in the range of the acceptable criteria of 100 ± 10 %, calibrations were carried out with PPs standards dissolved in pure solvent [36]. As summarized in Table 4.2, good linearity was found for all compounds with correlations coefficients higher than 0.99 [20].

Table 4.2 - Limits of detection (LOD) and quantification (LOQ), linearity (correlation coefficient, r^2) and matrix effect (ME).

Compound Name	LOD (ngg^{-1},dry weight)	LOQ (ngg^{-1},dry weight)	Correlation coefficient (r^2)	ME (%)	
				5 ngg^{-1}	25 ngg^{-1}
Trifluralin	1.1	2.1	0.9989	101	100
α-HCH	1.0	2.0	0.9979	91	98
HCB	0.2	0.5	0.9984	109	101
β-HCH	1.0	2.0	0.9993	105	102
γ-HCH	0.5	1.0	0.9986	100	100
Quintozene	0.6	1.2	0.9990	102	101
δ-HCH	1.0	2.0	0.9993	104	105
Heptachlor	0.5	2.0	0.9987	107	103
Aldrin	1.0	2.0	0.9982	99	102
Chlorpyrifos	1.0	2.1	0.9990	108	104
Isodrin	0.5	1.0	0.9991	107	97
Heptachlor epoxide	0.7	2.0	0.9986	106	103
γ-Chlordane	3.2	6.0	0.9986	99	103
α-Endosulfan	1.0	3.0	0.9991	106	103
Dieldrin	1.0	2.0	0.9991	104	103
p,p'-DDE	1.0	2.0	0.9987	104	102
Endrin	1.0	2.0	0.9990	106	104
p,p'-DDD	1.0	2.0	0.9988	103	103
p,p'-DDT	0.7	2.0	0.9992	106	107
Bromopropylate	1.3	2.4	0.9993	104	105
p,p'-Methoxychlor	2.0	5.0	0.9994	105	109
Mirex	1.2	2.5	0.9992	101	101

4.3.2.3 Accuracy and precision

Accuracy was evaluated by means of pesticides recoveries at three different levels of spiking concentration (Table 4.3). Recoveries in the range of 72 - 106% were found for the studied PPs in accordance with the established criteria of pesticides quality control reference guidelines (70 – 120%) [20]. Method performance was evaluated by comparison with a reference method that uses soxhlet extraction and column chromatography as sample clean-up prior to GC-ECD analysis [16-18]. As shown in Table 4.3, better recoveries

were found for a higher number of compounds when the extraction was carried out by SPLE. Precision, expressed as relative standard deviation (RSD), was also estimated using the replication of the recoveries tests. An average value of the three spiking levels was calculated and reported in Table 4.3. The obtained RSD are well below 20% being in good conformity with the quality control reference guidelines [20].

Table 4.3 Recoveries of priority pesticides from *Ulva* sp. samples by SPLE and soxhlet extraction prior to GC-ECD. SPLE was carried out at low, medium and high level of spiking. Values are reported as mean ± standard deviation (n = 5).

***Ulva* sp.**	**SPLE**				**Soxhlet**	
	Low level 5 ngg^{-1}	**Medium level 25 ngg^{-1}**	**High level 100 ngg^{-1}**		**100 ngg^{-1}**	
Priority Pesticide	**Recovery (%)**	**Recovery (%)**	**Recovery (%)**	**Mean RSD (%)**	**Recovery (%)**	**Mean RSD (%)**
Trifluralin	80 ± 1	75 ± 1	102 ± 2	3	-	
α-HCH	79 ± 8	76 ± 4	101 ± 1	5	67 ± 5	3
HCB	77 ± 2	53 ± 4	78 ± 1	3	-	-
β-HCH	98 ± 11	101 ± 2	100 ± 2	6	87 ± 4	5
γ-HCH	89 ± 12	87 ± 4	102 ± 1	7	76 ± 1	1
Quintozene	73 ± 2	72 ± 1	94 ± 2	5	61 ± 2	3
δ-HCH	87 ± 6	104 ± 1	107 ± 1	3	86 ± 7	8
Heptachlor	87 ± 9	77 ± 1	104 ± 1	5	69 ± 8	12
Aldrin	71 ± 1	72 ± 1	92 ± 4	3	-	-
Chlorpyrifos	94 ± 10	96 ± 3	102 ± 1	6	-	-
Isodrin	80 ± 1	90 ± 6	90 ± 4	4	-	-
Heptachlor epoxide	94 ± 4	92 ± 1	106 ± 1	2	-	-
γ-Chlordane	85 ± 2	82 ± 8	106 ± 1	2	-	-
α-Endosulfan	74 ± 1	80 ± 1	101 ± 2	1	-	-
Dieldrin	87 ± 1	99 ± 1	101 ± 1	3	-	-
p,p′-DDE	93 ± 9	97 ± 1	102 ± 2	5	102 ± 13	13
Endrin	91 ± 4	102 ± 4	104 ± 1	2	-	
p,p′-DDD	88 ± 3	98 ± 4	102 ± 3	1	92 ± 6	7
p,p′-DDT	98 ± 3	90 ± 1	104 ± 5	2	80 ± 11	13
Bromopropylate	84 ± 4	105 ± 3	98 ± 1	3	-	-
p,p′-Methoxychlor	91 ± 8	97 ± 2	97 ± 2	4	-	-
Mirex	86 ± 4	105 ± 3	103 ± 5	2	94 ± 13	14

4.3.2.4 Field application

The proposed method was applied to samples collected in the Bom Sucesso branch of Óbidos Lagoon. No PPs pesticides were detected in a sample collected during the summer season. However, some PPs and their metabolites were found in the *Ulva* sp. seaweeds collected in winter after a prolonged period of heavy rains. As shown in Table 4.4, the concentrations of the detected PPs were in the range of 3 to 11 ngg^{-1} in a dry weight basis (dw). Among them, higher concentrations were observed for those compounds with Log K_{ow} < 5 [13, 37]. Lindane (γ-HCH) presented a concentration of 11 ngg^{-1} above the MRL (10 ngg^{-1}). This can be an indication that care should be taken when wild seaweeds are collected for human consumption after heavy rain periods. The results suggest that *Ulva* sp. seaweeds tend to accumulate hydrophobic pesticides especially after a long period of storm events and have the potential to be used as early alert signals of aquatic pollution in such weather conditions. As referred by B. Pavoni et al. [4] the accumulation of some micro-pollutants in *Ulva* seaweeds can be linked to the large leaf-shaped morphology and to their lower bathymetric distribution. These characteristics allow them to be directly and in a longer contact with the contaminated runoff water. *Ulva* sp. seaweeds are abundant and easily to sample when compared to suspended particular matter (SPM). They are a promising analytical matrix for the evaluation of pesticides contamination by runoff waters.

Table 4.4 - Concentration (ngg^{-1}, dry weight base) of priority pesticides in a sample collected in Óbidos Lagoon after a period of heavy rains.

Priority Pesticide	Concentration (ngg^{-1})
α-HCH	10
β-HCH	7
γ-HCH (lindane)	11
δ-HCH	4
Heptachlor epoxide	7
α-Endosulfan	6
Dieldrin	5
p,p'-DDE	3
Endrin	4
p,p'-DDD	4
p,p'-DDT	5
p,p'-Methoxychlor	<LOQ

4.4. Conclusions

A fast and reliable analytical method based on the SPLE was developed for the screening of PPs in *Ulva* sp. seaweeds using GC-ECD. Method performance was in good agreement with the control quality guidelines for pesticides residues analysis. Better performance for a high number of pesticides was achieved by SPLE than by the traditional and most used Soxhlet extraction. The screening of PPs in *Ulva* sp. seaweeds was shown to be less time-consuming when the developed SPLE methodology was applied. The validated method was successfully applied to *Ulva* sp. seaweeds collected from a Portuguese coastal lagoon. The founded results suggest that *Ulva* sp. seaweeds tend to accumulate some PPs especially after a long period of storm events. This type of macroalgae are thus a promising analytical matrix for the evaluation of pesticides contamination by runoff waters working as early alert signals of aquatic pollution.

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5

Pathways of Priority Pesticides in Sediments of Coastal Lagoons: The Case Study of Óbidos Lagoon

Highlights

- Lagoon hydrodynamics influenced the dispersion and accumulation of PPs.
- Sediment monitoring is essential to control recently inputs of pesticides.
- Past application of lindane and DDT are still an ecotoxicological threat.

❖ M. I. Pinto, C. Vale, G. Sontag, J. P. Noronha, Pathways of priority pesticides in sediments of costal lagoons: The case study of Óbidos Lagoon, **Mar. Pollut. Bull.**, submitted.

Abstract

In this study the levels and the dispersion of the priority pesticides (PPs) in the sediments of a shallow Portuguese coastal lagoon were evaluated. Concentrations of PPs in surface and sediment core were reported. Results show that the PPs are confined to one part of the lagoon. Major sources of PPs inputs are from surface runoff of the surrounding agricultural fields and by discharge of small tributaries. Past and recently applied PPs were found at levels between 0.05 to 6.85 ng/%Al. The PPs risk assessment based on the ''probable effect level'' (PEL) show no biological effects in either sediments or aquatic organisms of Óbidos Lagoon, except for lindane, DDT and heptachlor epoxide.

Keywords: Pesticides; Coastal lagoons; Runoff; Water Framework Directive; Sediments; Ecotoxicological risks.

5.1. Introduction

Plants, animals and humans are vulnerable to thousands of diseases caused by bacteria, viruses, fungi, algae, nematodes, insects, etc. [1]. This implies a massive application of pesticides that have many benefits however, depending on their toxicity and if not properly applied they can pose many risks [1, 2]. In the European Union (EU) the pesticides are legislated through many directives depending on the sector they are applied. Directive 2000/60/EC [3], also known as Water Framework Directive (WFD), is a good example concerning the good quality status of surface waters. It covers fresh, transitional and coastal waters. WFD requires the progressive reduction and/or phase-out of some toxic and persistent substances defined as priority substances (PSs) and priority hazard substances (PHSs) [3]. Organochloride pesticides like DDT and lindane among others are included in the list of the PSs published under Annex X of WFD [3]. Most of them are extremely toxic and are known by their persistency and high tendency to accumulate in sediments and biologic tissues [3, 4]. Most of the priority pesticides (PPs) have been banned, nevertheless they are still a reality in Portuguese soils [5]. PPs can easily enter into coastal lagoons, estuaries and coastal waters from human activities like agricultural, urban discharges and industries. Heavy rains and runoff facilitate the discharge of these compounds. In Portugal, Óbidos Lagoon is of considerable ecological and economical interest. Despite its importance few studies were performed concerning pesticide screening. The aim of this study was thus to evaluate the concentration of the PPs in sediments of the upper part of the lagoon where fine particles from the watershed settle and to assess possible ecotoxicological risks.

5.2. Materials and methods

Surface sediment samples (0 – 2 cm) were collected in November of 2013 at 14 stations located in the upper part of Óbidos Lagoon (Fig. 5.1). In addition a 50-cm long sediment core was sampled at station 3. The core was

sliced at 2-cm intervals. After collection, all samples were air dried during 48 h, sieved to remove stones and gravel (2 mm mesh sieve), homogenized and stored at room temperature for chemical analyses [6, 7].

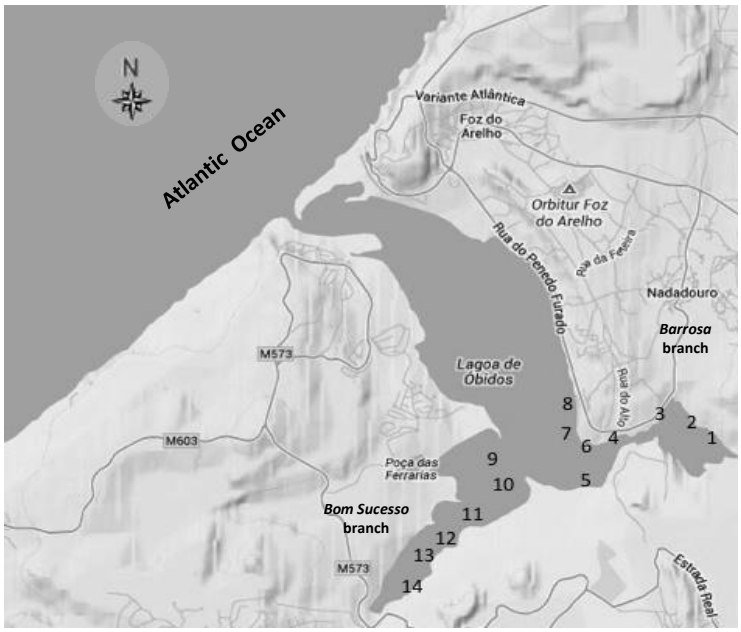

Fig. 5.1 – Location of the sediment sampling sites in Óbidos Lagoon.

The PPs listed in Table 5.1 were determined by gas-chromatography mass spectrometry (GC-MS) as described by Pinto et al. [8]. Aluminum was quantified by flame atomic absorption spectrometry according to Caetano et al. [9]. Total organic carbon (TOC) was determined by combustion with a CHN analyzer (CHN Fisons NA 1500 Analyzer, Fisons Instruments, Italy) [9]. For quality control 3 certified reference materials (CRM) were used. The CRM 1646a (estuarine sediment) and 1941b (organics in marine sediments) were purchased from the National Institute of Standards and Technology (Gaithersburg, USA) and the CRM PACS-2 (marine sediment) was purchased from the National Research Council Canada (Ottawa, Canada).

5.3. Results

Aluminum and total organic carbon in surface sediments ranged within broad intervals, 0.51 – 6.31% and 0.03 – 4.58%, respectively (Table 5.1). Good correlations (r^2=0.86) were found between these two parameters. The priority pesticides (PPs) α-hexachlorocyclohexane (α-HCH), hexachlorobenzene, quintozene, isodrin, dieldrin, *p,p'*-DDD and *p,p'*-DDT showed good correlation to Al (r^2>0.96). Table 1 gives the concentration of the quantified PPs normalized to Al in 14 sediments samples from the upper part of Óbidos Lagoon. Chlorpyrifos, hexachlorobutadiene, *p,p'*-DDE were quantified in most of the samples and all the analyzed pesticides were found in the three sites (1, 2 and 3) located in a confined inner bay of the upper lagoon. Among the isomers of hexachlorocyclohexane (HCH) higher concentrations were found for γ-HCH also known as lindane. Endosulfan sulfate, which is a metabolite of α-endosulfan and β-endosulfan occurred only in one part of the lagoon at concentrations below its parent compounds. The concentration of α-endosulfan was higher than β-endosulfan. Heptachlor and its metabolite heptachlor epoxide are also present in the lagoon. In each site heptachlor epoxide is approximately half of the content of heptachlor. The same behavior was found for aldrin and its epoxide dieldrin. In general, the metabolite *p,p'*-DDE shows lower concentrations in sites where *p,p'*-DDT and *p,p'*-DDD were not present (Fig. 5.2). A DDT ratio of 0.7 ((*p,p'*-DDE + *p,p'*-DDD)/ ΣDDT's) was found for all stations where *p,p'*-DDT was present. Chlorfenvinphos showed the highest concentrations.

Table 5.1 – Aluminum (%), total organic carbon (%) and concentration of priority pesticides (PPs) normalized to Al (ng/%Al, dry weight) in surface sediments of Óbidos Lagoon.

	Sites and respective normalized PPs concentration (ng/%Al)													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Al (%)	5.96	6.01	6.31	1.34	2.35	3.99	1.02	0.51	3.77	5.71	5.67	5.91	5.77	6.28
C_{org} (%)	3.04	4.58	2.13	0.03	0.58	1.34	0.04	0.06	2.91	1.96	1.96	1.63	2.34	1.75
Priority Pesticide (CAS)*														
Hexachlorobutadiene (87-68-3)	0.92	1.42	1.76	*	*	*	*	*	*	0.05	0.08	0.14	0.09	0.10
Trifluralin (1582-09-8)	0.58	0.68	0.72	*	*	*	*	*	*	*	*	*	*	*
α-HCH (319-84-6)	0.93	1.04	1.55	*	*	*	*	*	*	*	*	*	*	*
Hexachlorobenzene (118-74-1)	1.75	1.80	2.27	*	*	*	*	*	*	*	*	*	*	*
γ-HCH (58-89-9)	2.25	*	*	*	*	*	*	*	*	*	*	*	*	*
Quintozene (82-68-8)	0.89	0.96	1.19	*	*	*	*	*	*	*	*	*	*	*
δ-HCH (319-86-8)	*	1.34	*	*	*	*	*	*	*	*	*	*	*	*
Heptachlor (76-44-8)	1.15	1.73	1.81	*	*	*	*	*	*	*	*	*	*	*
Aldrin (309-00-2)	0.89	0.70	1.13	*	*	*	*	*	*	*	*	*	*	*
Chlorpyrifos (2921-88-2)	1.91	1.93	2.27	1.67	1.22	0.82	*	*	0.43	0.35	0.49	0.60	0.47	0.35
Isodrin (602-050-00-4)	1.53	1.83	2.52	*	*	*	1.21	2.55	*	*	*	*	*	*
Heptachlor epoxide (1024-57-3)	0.54	0.65	0.83	0.31	*	*	*	*	*	*	*	*	*	*
Chlorfenvinphos (470-90-6)	5.73	6.85	5.64	*	*	*	*	*	*	*	*	*	*	*
Chlordane (57-74-9)	0.62	0.72	0.72	*	*	*	*	*	*	*	*	*	*	*
α-Endosulfan (959-98-8)	0.98	1.59	1.84	*	*	*	*	*	*	*	*	*	*	*
Dieldrin (60-57-1)	0.37	0.48	0.68	*	*	0.07	0.34	*	*	*	*	*	*	*
p,p'-DDE (72-55-9)	1.07	1.14	1.62	0.30	0.37	0.21	0.77	1.20	0.43	0.29	0.30	0.34	0.33	0.21
Endrin (72-20-8)	1.88	2.38	2.63	*	*	*	*	*	*	*	*	*	*	*
β-Endosulfan (33213-65-9)	0.68	1.13	1.39	*	*	*	*	*	*	*	*	*	*	*
p,p'-DDD (72-54-8)	0.64	0.72	1.15	*	*	*	*	*	*	*	*	*	*	*
Endosulfan sulphate (1031-07-8)	*	*	1.23	*	*	*	*	*	*	*	*	*	*	*
p,p'-DDT (50-29-3)	0.81	0.89	1.18	*	*	*	*	*	*	*	*	*	*	*
p,p'-Methoxychlor (72-43-5)	1.34	1.15	1.93	*	*	*	*	*	*	*	*	*	*	*
Mirex (2385-85-5)	1.02	0.90	1.02	*	*	*	*	*	*	*	*	*	*	*

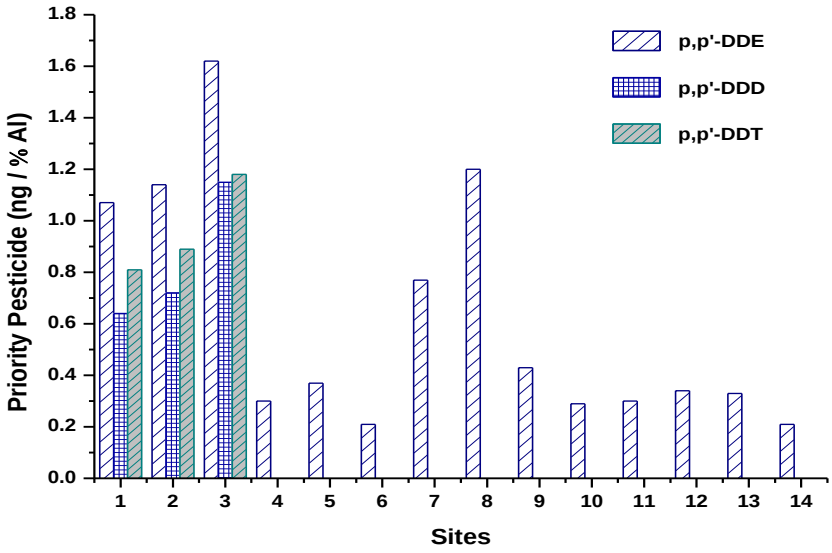

Fig. 5.2 – Distribution of *p,p'*-DDT and its metabolites *p,p'*-DDD and *p,p'*-DDE in Óbidos Lagoon. The concentrations were normalized relatively to Al (%).

In the sediment core the following PPs were quantified: hexachlorobutadiene (HCDB), γ-hexachlorocyclohexane or lindane (γ-HCH), *p,p'*-DDT and its metabolites *p,p'*-DDE, and *p,p'*-DDD, β-endosulfan and *p,p'*-methoxychlor. Most of the PPs present a maximum of concentration at two different zones of the core as shown in Fig. 5.3a for lindane. The pesticide β-endosulfan was an exception (Fig. 5.3b). This compound together with *p,p'*-methoxychlor have a higher fluctuation exhibiting high concentrations below the 14 cm of depth. The pesticide *p,p'*-DDT showed a profile similar to γ-hexachlorocyclohexane and was completely absent below 26 cm of depth. A DDT ratio ((*p,p'*-DDE + *p,p'*-DDD)/ ΣDDT's) higher than 0.5 was found above 34 cm of depth. Below that zone *p,p'*-DDD was the dominant metabolite. No correlations were found between the Al and TOC content and between each detected PPs and these parameters.

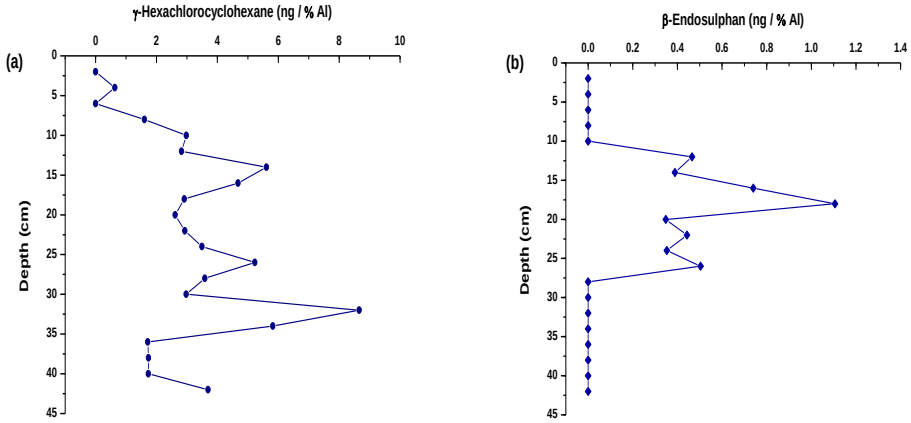

Fig. 5.3 – Depth profile of the PPs: (**a**) γ-hexachlorocyclohexane or lindane (γ-HCH), (**b**) β-endosulphan. Concentrations were normalized relatively to Al (%).

5.4. Discussion

The good correlations between the Al and TOC content is an indication that organic matter in sediments of the upper lagoon is associated with fine particles, which is in accordance with previous studies [10]. Erosion of agriculture soil and degradation of macroalgae biomass covering the lagoon floor may contribute to the high organic matter. The presence of PPs in the upper Barrosa branch, a confined semi-enclosed area with weak currents, reflects local sources and conditions favouring the particle deposition. In fact, most of the PPs are highly hydrophobic with a high tendency to be sorbed by the fine particles. Volatilization favours the low values or absence of organophosphate chlorpyrifos and cyclodiene pesticides like aldrin, isodrin, endosulfan, heptachlor, chlordane and also quintozene and hexachloro-butadiene [11-13]. The presence of chlorpyrifos in the upper branches of the lagoon may be a consequence of recent application in the surrounding fields. Chlorpyrifos is commonly applied as an insecticide in pome fruits, vines and vegetables of the region [14]. Chlorfenvinphos might also be present due to recently applications. Both organophosphate PPs have a high tendency to

hydrolyze at high pH with a half-life of 17 days (pH 9.1, 20 ºC) for chlorfenvinphos and 100 days (phosphate buffer, pH 7, 15 ºC) for chlorpyrifos [12]. Although the hydrolysis rate can be affected by the strong adsorption to sediments, both compounds are less persistent than the other PPs but still highly toxic to aquatic organism [12, 15]. Chlorfenvinphos is an insecticide that has been used in public health in the control of mosquito larvae and also in animals as an ectoparasiticide. One of the main industrial pressures in the zone is livestock [14]. Cal River is a tributary that discharges in the Barrosa branch with a passage by rural areas and urban areas. Hexachlorobutadiene (HCBD) is an organochloride pesticide and is more persistent than the organophosphate PPs. HCBD was used as intermediate in chemical industry (solvent for rubber and other polymers) and also as fumigant for treating grapes [16]. HCBD is regarded as a priority hazardous substance thus it is subjected to stepwise cessation or phasing out of discharges, emissions and losses [16]. The presence of HCDB in the two branches of OL can be due to soil runoff as well as also due to discharge of potentially contaminated small tributaries that enter into the lagoon. HCBD together with chlorpyrifos, hexachlorobenzene and γ-hexachlorocyclohexane have been detected (not quantified) in waters of the Western river basin where OL is included [14]. In the core, HCBD shows concentrations in the range 0.23 – 0.55 ng/%Al. Although the concentrations are lower than in the surface these results point out for an extensive use in the past. HCBD has a high Henry's Law constant indicating volatilization from wet surfaces, such as the inter-tidal sediments exposed to the atmosphere around low tide. Volatilization may however be counterbalanced by the high tendency to be sorbed by organic matter. The compound does not hydrolyse due to lack of hydrolysable functional groups and data on photolysis is limited. There is evidence that HCBD is not readily biodegraded. However, findings on degradation pathways are still controversy [16].

Contrary to chlorpyrifos and HCBD, the PPs hexachlorobenzene and γ-hexachlorocyclohexane (γ-HCH) were detected only in the confined inner

Barrosa branch. Hexachlorobenzene was used as a fungicide in seed treatment and there are no commercial uses of the pesticide, nevertheless it can still be released from certain thermal processes in the metallurgical industry, oil refinery and also motor vehicles [17]. Lindane isomers α-HCH and δ-HCH were also present. Although the insecticidal activity is mainly due to lindane its isomers are also toxic [18]. HCH is produced by photochemical chlorination of benzene [18]. The technical-grade HCH usually contains: α-HCH (60 – 70%), β-HCH (5 – 12%), γ-HCH (10 – 15%), δ-HCH (6 – 10%) and ε-HCH (3 – 4%) [18]. Refined γ-HCH also known as lindane is more expensive and contains only trace amounts of the others isomers [12, 18]. Thus, the presence of α-HCH and δ-HCH is an indication of application of technical-grade HCH. The higher concentrations of γ-HCH comparatively to α-HCH can be caused by application of refined lindane superimposed on a background of technical-grade HCH. In the sediment core, none of the isomers was detected suggesting an extensive application of lindane in the past and use of technical-grade HCH more recently. Both formulations are not authorized in the EU for agricultural proposes. Only lindane can be used for public health (scabies and lice) and as veterinary topical insecticide [19]. The β-HCH is the most stable of the isomers, however, the compound was not detected in any of the stations. α-HCH in conjunction with hexachlorobenzene, quintozene, isodrin, dieldrin, *p,p'*-DD and *p,p'*-DDT show positive correlation with %Al, indicating the effect of particle size on the partition of those PPs in sediments [17]. It is also possible that the bioavailability towards aquatic invertebrates might be higher in case of suspended sediments [20]. The insecticide endosulfan is a mixture of two stereoisomers α-endosulfan and β-endosulfan with a technical-grade percentage in the range of 64 – 67% for the first stereoisomer. Endosulfan has been used as an insecticide in fruits, vines, olives, vegetables and in the control of tsetse flies [12]. Its metabolite endosulfan sulfate is more persistent. The concentration of endosulfan sulfate is lower but in the same range of its parent isomers suggesting an aged application of the starting compound. In the core,

only β-endosulfan was detected. Many bacteria and fungi have been reported to be endosulfan degraders [21]. Degradation of endosulfan is pH dependent increasing at high pH (8.5) due to an increased growth and activity of bacteria in alkaline conditions [21]. Pure culture studies revealed that *Staphylococcus sp*. utilized more β-endosulfan compared to α-endosulfan while *Bacillus circulans-I* and *Bacillus circulans-II* utilized more α-endosulfan than β-endosulfan [21]. The presence of only β-endosulfan can be thus a consequence of a highly and selective biodegradation of α-endosulfan by certain microorganisms. Degradation of endosulfan can occur by attack on sulfite group by oxidation and or hydrolysis to form the toxic endosulfan sulfate and the non toxic endosulfan diol, respectively [21]. Kwon et al. [22] reported that endosulfan diol is the major metabolite as pH increases whereas endosulfan sulfate is the major breakdown product as pH decreases. However at sub-surface of the sediment sulphate is most likely used as oxidant for the oxidation of organic matter. This fact may explain the absence of endosulfan in the layers of the sediments. At the surface, endosulfan seems to follow an oxidative pathway. Relatively to heptachlor and aldrin, their respective breakdown products heptachlor epoxide and dieldrin show an average ratio of metabolite/parent pesticide higher than 0.3 suggesting a past input of both PPs. Isodrin and its epoxide endrin are the least stable of the cyclodienes pesticides and the ones exhibiting higher concentrations. Isodrin and endrin showed to be stable in formulations with basic reagents [23]. The pH of the sediments of OL is between 7 and 8 [24] and the presence of other alkaline substances might have a stabilizing effect on cyclodienes [23, 24]. Nevertheless, further work needs to be done to evaluate the stability of such compounds in marine sediments.

The pesticide *p,p'*-DDT was detected in sediments of station 1 to stations 3 of Barrosa branch although its main metabolite *p,p'*-DDE was detected in all stations suggesting a leveling off in *p,p'*-DDT inputs along the lagoon. A DDT ratio ((*p,p'*-DDE + *p,p'*-DDD)/ ΣDDT's) higher than 0.5 can be interpreted as a past and weathered input of p,p'-DDT [15]. The higher levels of *p,p'*-DDE might

be caused by either biological or photochemical transformation. In marine sediments, and under laboratory conditions, *p,p'*-DDT has been degraded into *p,p'*-DDD and *p,p'*-DDE under anaerobic and aerobic conditions, respectively [11]. As shown in Fig. 2, the concentration of *p,p'*-DDE in Barrosa branch (station 1 to 3) was higher than *p,p'*-DDD. Although the sediments of the Barrosa branch have shown to be reductive, the results suggest possible DDT degradation through aerobic processes [17, 24]. In core, the results confirm past inputs of DDT due to high concentrations of *p,p'*-DDE. Results point out for predominantly reductive conditions below 34 cm of depth since *p,p'*-DDD > *p,p'*-DDE. The pesticide *p,p'*-methoxychlor was also present in surface sediments of the inner Barrosa branch. The high concentrations in the core below 14 cm are an indication of high inputs in the past. Methoxychlor was formulated to substitute the insecticide DDT. The compound has been released to the environment mainly as a result of its application to crops and livestock [25]. Studies show that *p,p'*-methoxychlor can be biodegraded under either aerobic or anaerobic conditions however, limited data is available in marine sediments. Future analysis of *p,p'*-methoxychlor degradation products like 1,1-dichloro-2,2-di(4-methoxy-phenyl)ethane (DMDE) and Dimethoxy-diphenyldichloroethane (DMDD) would be an important tool in the interpretation of its degradation in marine sediments [25].

To evaluate the possible ecotoxicological risks of the PPs in the studied area their concentrations were compared with the Environmental Quality Standards (EQSs) established by the Italian Parliament and also by the ''probable effect level'' (PEL) defined in the Canadian environment quality guidelines for marine sediment [15, 26, 27]. The PEL are defined as the level above which adverse effects on aquatic biota are expected to occur frequently [15]. The EQSs were established for aldrin, dieldrin, α-HCH, β-HCH, lindane DDT, DDD, DDE and hexachlorobenzene [26]. The PEL were defined for endrin, dieldrin, lindane, heptachlor epoxide, DDT, DDE, DDD and chlordane [15]. Results show PPs concentrations above the EQSs. Nevertheless, the concentrations are below

the limit to show biological effects in either sediments or aquatic organisms of OL, except lindane, DDT and heptachlor epoxide.

5.5. Conclusions

Priority pesticides (PPs) do not occur naturally. Most of them have been restricted or banned in the EU. Because of its widespread application in the past, PPs were detected in Óbidos Lagoon. Their occurrence was mainly in one of the inner bays of the lagoon as a consequence of its hydrodynamic conditions. PPs inputs, especially lindane, DDT and heptachlor must be kept under control in the future. Results show that sediment monitoring is essential for the control of recently inputs of PPs as well as to understand and predict their transport, environmental fate and effect on water quality and on aquatic organisms. Sediments are important matrix for PPs monitoring and EQSs for the entire list of PPs should be established by all Member States.

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6

Influence of Dissolved Organic Matter on the Photodegradation and Volatilization Kinetics of Chlorpyrifos in Coastal Waters

Highlights

- Photolysis rate of chlorpyrifos in saline waters is enhanced by high inputs of DOM.
- Saline waters: photolysis and volatilization can be major transformation processes.
- Medium pressure Hg lamps are more efficient than other UV irradiation sources.
- The main degradation product of chlorpyrifos was 3,5,6-trichloro-2-pyridinol.

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Abstract

Irradiation by a medium-pressure mercury (Hg) lamp was used to study the photodegradation of chlorpyrifos (diethyl-*O*-3,5,6-trichloropyridin-2-yl phosphorothioate) in salt, deionized water (unbuffered), and a natural saline water. The UV irradiation produces 3,5,6-trichloro-2-pyridinol (TCP) with accelerated photodegradation kinetics of the parent compound. The results show that chlorpyrifos photolysis followed pseudo-first-order kinetics in the presence and absence of salt with no significant difference between the photodegradation rates in saline waters and unbuffered deionized water. Addition of Suwannee River natural organic matter (SRNOM) to mimic the mixing of freshwaters with seawaters significantly changed the photodegradation rate constant of chlorpyrifos in saline waters. The influence of hydrolysis and volatilization were also evaluated. While photolysis was found to be the main transformation process in unbuffered deionized water, both volatilization and photolysis can be important pathways of chlorpyrifos loss in natural saline waters and under aerated conditions.

Keywords: Chlorpyrifos; Pesticides; Dissolved organic matter (DOM); Photolysis; UV irradiation; Salinity.

6.1. Introduction

Chlorpyrifos (diethyl-*O*-3,5,6-trichloropyridin-2-yl phosphorothioate) is a broad spectrum insecticide, acaricide and nematicide [1] used in the control of a broad array of arthropod pests. It is primarily a contact insecticide but has some efficacy through ingestion [1, 2]. It is applied to soil or to foliage in a wide range of crops, including corn, pome fruit, vines, vegetables, etc. [1, 2]. Application can be carried out at different times during the growth and dormant seasons [2]. When applied as recommended the compound is non-phytotoxic to most plant species [3]. However, chlorpyrifos, like other organophosphate pesticides, is very toxic to vertebrate animals with an acute oral LD_{50} for rats between 60 to 135 $mgkg^{-1}$ [3, 4]. Due to its toxicity the compound was classified by the European Commission as a priority substance with an annual average Environmental Quality Standard (EQS) set at 30 ngL^{-1} for surface waters [5]. In spite of the efforts that have been taken to limit its use, higher concentrations above its EQS have been found in both aquatic environments and wastewater effluents [4, 6-8].

Chlorpyrifos has a low water solubility (<2 mgL^{-1}) and a strong tendency to be sorbed by organic matter [9]. In soils, the degradation process is mainly governed by biotic factors, while in water the abiotic processes, such as hydrolysis and photolysis, are thought to be major transformation pathways [2, 9-11]. Volatilization is also important in chlorpyrifos dissipation [8, 12]. This is especially true of its behavior on plant foliage where it is the major loss process [2, 8, 13, 14]. In water, volatilization is thought to be reduced due to partitioning with suspended solids and deposition onto bottom sediments [8]. The mechanisms of chlorpyrifos degradation in several environmental compartments have been established [2, 9, 10]. The compound, 3,5,6-trichloro-2-pyridinol (TCP), is the single and most important degradation product in soils and in water (Fig. A1) [2, 8, 15, 16]. The intermediate chlorpyrifos oxon is difficult to detect as it is very unstable and readily transformed to TCP by hydroxylation [17]. The reversible formation of

3,5,6-trichloro-2-methoxy-pyridine (TMP) from TCP is dependent on biological activity of soils [2, 18]. Desethyl chlorpyrifos can be formed by hydrolysis of chlorpyrifos at high pH or by the action of glutathione-S-transferase enzymes [15, 16].

Chlorpyrifos is one of the most widely applied of the organophosphate pesticides, and its behavior in the environment has been extensively characterized [2, 9, 19]. However, little information is available on its transformation processes in saline environments and less data is available for its behavior in water and sediments when compared to soils [2]. Moreover, the influence of dissolved organic matter on chlorpyrifos fate has been limited to sorption mechanisms [20].

Many water and wastewater treatment plants are now using photochemical technologies to comply with governmental regulations for water quality standards. Low-pressure mercury lamps (LP Hg lamps) find particular application in the disinfection of air, water and wastewater treatment systems [21, 22]. In compact water treatment systems, medium-pressure mercury lamps (MP Hg lamps) have been reported as successful alternatives to the traditional LP Hg lamps [21]. Ultraviolet (UV) radiation delivered by MP Hg lamps in combination with other oxidants and/or catalysts has also been shown to be effective in the degradation of pesticides [23]. However, to the best of our knowledge, no studies have been reported concerning the photodegradation of chlorpyrifos by MP Hg lamps in saline waters. This is an important omission when considering the fate and transport of chlorpyrifos in waters which discharge to estuaries lagoons, or the ocean. Therefore, the aim of the present study was: (a) to investigate the photodegradation kinetics of chlorpyrifos in unbuffered deionized, saline water and a natural estuary water using a system with a MP Hg lamp; (b) to investigate the impact of dissolved organic matter on the photodegradation rate of chlorpyrifos and (c) to evaluate the influence of other light

independent transformation processes, such as hydrolysis and volatilization, on the overall loss of chlorpyrifos.

6.2. Materials and methods

6.2.1. Reagents

Acetonitrile (HPLC grade) was purchased from Carlo Erba (Rodano, Italy) and ethyl acetate (HPLC grade) from Sigma-Aldrich (St. Louis, MS, USA). *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, ≥97%) was obtained from Fluka (St. Louis, MS, USA). Sodium chloride p.a. (NaCl) was purchased from Riedel-de-Haen (Darmstadt, Germany) and hydrochloric acid (HCl) was obtained from Merck (Darmstadt, Germany). The reference material Suwannee River natural organic matter (1R101N) was obtained from the International Humic Substances Society (IHSS). Chlorpyrifos (99.9%) and the polydimethylsiloxane (PDMS) fibers were supplied by Sigma-Aldrich (St. Louis, MS, USA). SPE Oasis® HLB 3 cc (60 mg) extraction cartridges were supplied by Waters Corporation (Milford, MA USA). Chlorpyrifos stock solution (1000 mgL^{-1}) was prepared in acetonitrile. Dilutions (to 1.0 mgL^{-1}) were made in unbuffered deionized water (0.80 μScm^{-1}), salt water prepared in deionized water (NaCl 34.6 gL^{-1}) and a natural saline water collected from a Portuguese costal lagoon. Solutions were also made with the same type of waters to which Suwannee River natural organic matter (SRNOM) was added. Chlorpyrifos stock solution was kept at 4 ^{o}C and the corresponding diluted solutions were prepared on a daily basis. The concentration of iodine and bromide ions was determined by ionic chromatography and molecular absorption spectroscopy, respectively, with reagent grade chemicals as described elsewhere [24, 25].

6.2.2. Equipment

The chromatographic equipment consisted of an Agilent Technologies 6850 GC Network System with an Agilent 5975C VL MS detector (Agilent

Technologies, Waldbronn, Germany). Photolysis was performed with a Heraeus Noblelight model TQ 150 UV medium-pressure mercury vapour lamp (Heraeus Noblelight GmbH, Hanau, Germany). Deionized water (0.80 μScm^{-1}) was obtained from a Milli-Q50 water purification system (Merck Millipore, Darmstadt, Germany). A Palintest Photometer 5000 (Palintest Ltd., Gateshead, England) was used to characterize the collected natural saline water in terms of nutrients. UV-Vis analyses were carried out in a Spectronic Helios alpha dual-beam spectrophotometer (Thermo Scientific, Waltham, MA, USA).

6.2.3. Sampling

The natural saline water was collected in the Óbidos Lagoon. This western Portuguese coastal lagoon is a shallow aquatic system that has been subject of several studies including pesticide analysis [26-31]. The absence of chlorpyrifos in the collected sample was confirmed by gas chromatography-mass spectrometry (GC-MS) as described in the analytical procedure. The water was characterized in terms of pH, salinity and pollution indicators (Table 6.1).

Table 6.1 - Physicochemical properties and pollution indicators of the collected water sample from Óbidos Lagoon. Values are expressed and as mean values ± standard deviation (n = 3).

Parameter	Field Water
pH	8.27 ± 0.1
Salinity	30.0 ± 0.1
NO_3^- (mg N L^{-1})	0.73 ± 0.04
PO_4^{-3} (mg P L^{-1})	0.15 ± 0.01
NH_4^+ (mg N L^{-1})	0.46 ± 0.01
I^- (μgL^{-1})	50 ± 5
Br^- (mgL^{-1})	41 ± 1

6.2.4. Photolysis experiments

The photodegradation studies were carried out in a pear-shaped glass reactor fitted with a MP Hg lamp that emits radiation in the range of 200 to 400 nm [21]. The lamp was cooled to 18 ± 1 ^{o}C with tap water using a quartz-cooling jacket. Photolysis tests were carried out with 300 mL of chlorpyrifos solution at 1 mgL^{-1} prepared in: (a) unbuffered deionized water with and without the addition of SRNOM (20 mgL^{-1}), (b) saline water prepared with NaCl (34.6 gL^{-1}) with and without SRNOM (20 $mgL^{-1}$and 5 mgL^{-1}) and (c) natural saline water collected from Óbidos Lagoon with and without the addition of SRNOM (20 mgL^{-1}). Samples were irradiated for 60 min under aerated conditions with an airflow rate of 2 $Lmin^{-1}$. Subsamples (2 mL) were removed, over this period, for chlorpyrifos analysis by GC-MS. Control samples (2 mL of chlorpyrifos solution at 1 mgL^{-1}) were placed in the dark in a closed vial. All experiments were run in triplicate.

6.2.5. Volatilization experiments

Volatilization experiments were carried out in the same system (pear-shaped glass reactor) that was used for the photodegradation studies. The tests were made with 300 mL of chlorpyrifos solution at 1 mgL^{-1} prepared in (a) unbuffered deionized, (b) saline water prepared with NaCl (34.6 gL^{-1}) with and without the SRNOM at 20 mgL^{-1} and (c) natural saline water collected from Óbidos Lagoon. To evaluate the effect of aeration in chlorpyrifos dissipation, volatilization experiments were carried under aerated (air flow rate 2 $Lmin^{-1}$) and unaerated conditions for a period of 60 min. Over this period, subsamples (2 mL) were taken for chlorpyrifos analysis by GC-MS.

6.2.6. Analytical procedure

6.2.6.1 UV-Vis analysis

UV-Visible spectra were measured on a dual-beam spectrophotometer using 1 cm path length quartz cuvettes. Water samples were scanned in the

range of 200 to 600 nm. The spectra slopes in the range of 275 – 295 nm ($S_{275-295}$, nm^{-1}) were obtained from the regression line of the Naperian logarithm of the absorption coefficient (a) *vs.* wavelength λ. The absorption coefficient a (m^{-1}), at wavelength λ, was calculated from the relation:

$$a = 2.303 \times A(\lambda)/l \qquad (\mathbf{1})$$

where $A(\lambda)$ is the absorbance measured across the cell path length l (m).

6.2.6.2 Solid phase microextraction (SPME)

Chlorpyrifos was extracted from the aqueous solutions by solid phase microextraction (SPME) using a polydimethylsiloxane (PDMS) fiber for GC-MS analysis. The PDMS fiber was pre-conditioned as recommended by the manufacturer (30 min, 250 ^{o}C). Extractions were carried out by direct immersion of the fiber in vials with PTFE-lined screw caps with 2 mL of chlorpyrifos samples. The extractions were done at 700 rpm for 15 min at room temperature.

6.2.6.3 GC-MS/SIM analysis

Chlorpyrifos determination was carried out by gas chromatography-mass spectrometry (GC-MS). The separation was performed on a silica capillary column (HP-5MS, 30 m x 0.25 mm i.d.; d_f: 0.25 µm, Agilent-J&W Scientific) at a helium flow rate of 1 mL min^{-1}. The MS spectra were obtained using Electron Impact (EI) at 70 eV. The detector was operated under selected-ion monitoring (SIM) acquisition mode (Table 6.2). The ion source, transfer line, and the detector temperature were kept at 230 ^{o}C, 150 ^{o}C and 280 ^{o}C, respectively. The injector temperature was 250 ^{o}C and the GC gradient temperatures were: 100 ^{o}C (0.50 min), 20 ^{o}C/min to 260 ^{o}C (1.50 min). Data acquisition and data processing were carried out with the Agilent ChemStation software.

Table 6.2 - Mass spectrometry data from GC-MS/SIM analysis and some of physicochemical properties of chlorpyrifos [1, 6-8].

Retention time	8.09 min
m/z	197, 258, 314
Molecular mass	350.59 $gmol^{-1}$
Vapor pressure	0.88 – 2.7 × 10^{-3} Pa (25 °C)
Henry Law's constant (H´)	0.3 – 1.8 Pa m^3mol^{-1} (20-25 °C)
Log k_{ow}	3.31 – 5.27
CAS	2921-88-2

6.2.6.4 Extraction and derivatization of degradation products

Degradation products were extracted by solid phase extraction (SPE) and the compounds were derivatized by silylation with *N-tert*-butyl-dimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) prior to GC-MS analysis. Chlorpyrifos solutions (150 mL) taken after the photolysis experiments were acidified with 500 µL of 1 M HCl before SPE. Samples were then loaded onto the SPE Oasis® HLB cartridges preconditioned with 2 mL of methanol, 2 mL of deionized water and 2 mL of acidified water. The compounds were eluted with 5 mL of ethyl acetate. The resulting ethyl acetate solution was evaporated to dryness under a gentle stream of nitrogen. Derivatization of the extracted products was carried out in closed vials with 50 µL of MTBSTFA at 50 °C for 1 h [15, 21, 32]. The derivatized solution (1 µL) was injected into the GC-MS system operating under SIM and full scan mode.

6.2.7 Rate constants and half-life calculations

The rate constants (k) for degradation/dissipation of chlorpyrifos in saline and unbuffered deionized waters were calculated by application of Eq. (2):

$$\ln\left[\frac{C_t}{C_0}\right] = -kt \qquad (2)$$

where, t is the irradiation time, C_t is the residual concentration of target compound at time t and C_0 its initial concentration. Half-life times ($t_{1/2}$) were calculated using Eq. (3):

$$t_{1/2} = \frac{0.693}{k} \qquad (3)$$

6.3. Results and discussion

6.3.1 Photolysis kinetics

Complete photodegradation of chlorpyrifos was observed after 60 min of photolysis with an MP Hg lamp in both saline and unbuffered deionized water with and without the addition of SRNOM. In all cases (Fig. 6.1) the linear plots confirm that photodegradation of the chlorpyrifos follows pseudo-first order kinetic behavior.

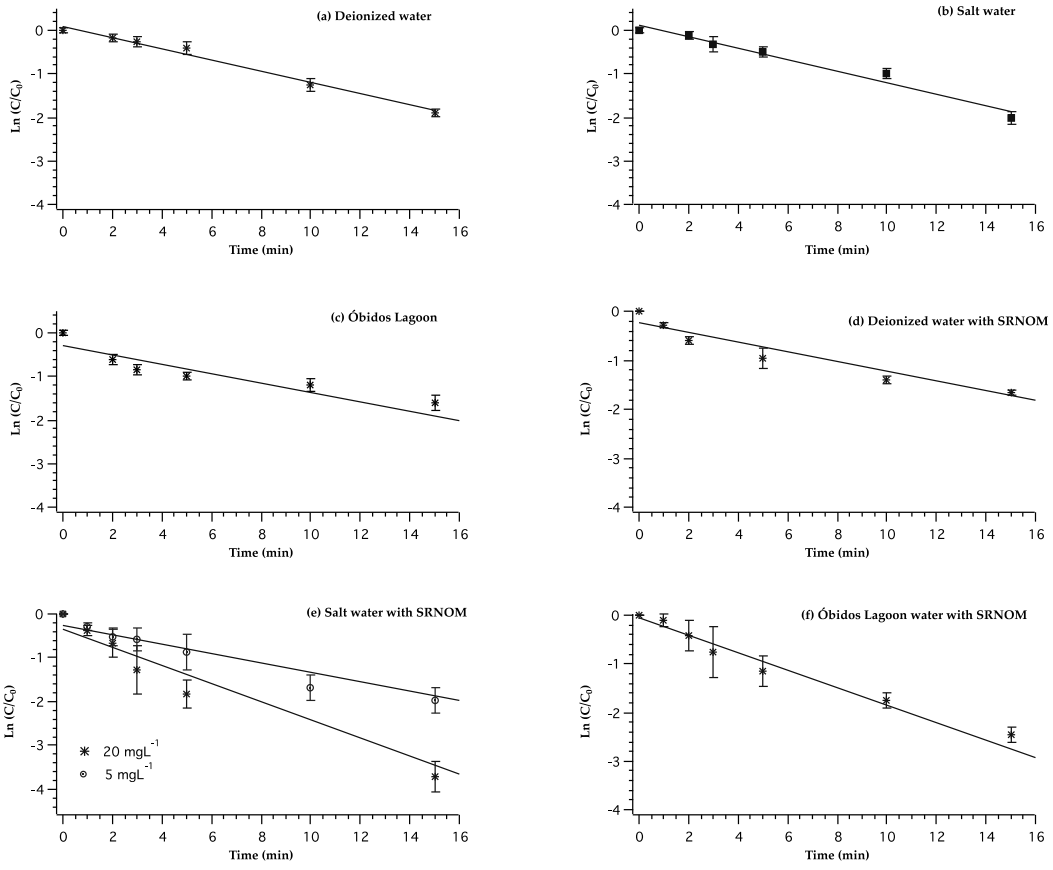

Fig. 6.1 - Photodegradation kinetics of chlorpyrifos under aerated conditions (airflow 2 $Lmin^{-1}$) in: (**a**) unbuffered deionized water; (**b**) salt water (34.6 gL^{-1} NaCl); (**c**) natural saline water taken from Óbidos Lagoon; (**d**) unbuffered deionized water enriched with SRNOM 20 mgL^{-1}; (**e**) salt water enriched with SRNOM 20 mgL^{-1} and 5 mgL^{-1}; (**f**) natural saline water taken from Óbidos Lagoon enriched with SRNOM 20 mgL^{-1}.

As shown in Table 6.3 the photodegradation rate constants were identical, within experimental error for salt, unbuffered deionized water and natural saline water (Óbidos Lagoon). The half-life times ($t_{1/2}$) were 5 min in salt water and 6 min in natural saline water and unbuffered deionized water. In contrast with the behavior on photodegradation of the pesticide fenarimol, no stabilizing effect was observed on chlorpyrifos photolysis in saline waters [33].

Table 6.3 – Photodegradation rate constants (k) and respective half-lives ($t_{1/2}$) of chlorpyrifos dissolved in unbuffered deionized water, salt water (34.6 gL^{-1}) and natural saline water with and without the addition of SRNOM to a final concentration 5 and 20 mgL^{-1}. Values are expressed and as mean values ± standard deviation (n = 3).

Water	k (min^{-1})	$t_{1/2}$ (min)	r^{2*}
Unbuffered deionized water	0.12 ± 0.01	5	0.97
Salt water	0.13 ± 0.01	5	0.98
Natural saline water	0.11 ± 0.02	6	0.94
Unbuffered deionized water with SRNOM (20 mgL^{-1})	0.10 ± 0.01	7	0.92
Salt water with SRNOM (5 mgL^{-1})	0.11 ± 0.03	5	0.96
Salt water with SRNOM (20 mgL^{-1})	0.21 ± 0.04	3	0.96
Natural saline water with SRNOM (20 mgL^{-1})	0.18 ± 0.03	4	0.94

* – r^2 is the correlation coefficient.

Results show that the addition of SRNOM to the unbuffered deionized water did and in salt water with a low concentration of SRNOM (5 mgL^{-1}) did not significantly change the photodegradation rate constant of chlorpyrifos (Table 6.3). However, the photodegradation rate constant was double when SRNOM was added at a concentration of 20 mgL^{-1} in salt waters (Table 6.3). As illustrated in Fig. 6.2, the absorption coefficient of the enriched SRNOM waters decreased over the 60 min of irradiation exposure. The corresponding spectra slope calculated over the wavelength range of 275 nm to 295 nm ($S_{275-295}$) increased with an increase of the irradiation time (Table 6.4). An exception was observed for the natural saline water. The addition of chlorpyrifos to the natural water increased the $S_{275-295}$ to 0.039 nm^{-1} as a consequence of the steepness of the absorption coefficient due to chlorpyrifos absorbance. As shown in Fig. 6.3 chlorpyrifos has two maxima of absorbance one and 231 nm

and the other one at 287 nm in the range of wavelengths used to calculate the spectral slope. The natural saline water without chlorpyrifos shows an $S_{275-295}$ of 0.023 nm^{-1} typical of seawaters [34]. $S_{275-295}$ has been recommended as a reliable proxy of photobleaching of chromophoric dissolved organic matter (CDOM) [35-37]. The differences in the slopes are more pronounced in waters with a high content of chloride ions suggesting that photobleaching reactions are probably more effective in seawater than in freshwaters. Grebel et al. [38] found out that the presence of seawater concentrations of chloride and bromide ions enhanced absorbance photobleaching reactions rates by almost 40% regardless the CDOM source or the presence or absence of carbonate ions. Variations in the ionic strength did not change the enhancement of photobleaching by halide ions. In a second study, Grebel et al. [39] found out that seawater halides reduced the rate of indirect photolysis of the 17β-estradiol by 90%. Approximately 70% of the observed decrease was associated with ionic strength effects and the remainder associated to halide specific effects. Halide promotion of CDOM chromophore photobleaching was shown to play a major role in the halide specific effect. In the present study the photodegradation rate constant did not change significantly in natural saline waters with a chloride concentration close to seawaters nor in the salt waters with the same concentration of chloride ions and enriched with small amounts of SRNOM. These findings suggested that at low concentration of SRNOM and in high concentration chloride waters chlorpyrifos is degraded mainly by direct photolysis with photobleaching as a dominant process of SRNOM transformation. However, at high concentration of SRNOM and in salt waters indirect photolysis can have a significant effect on chlorpyrifos degradation. The importance of the amount and the type of dissolved organic matter (DOM) on the steady-state concentration of intermediate radicals was reported by Timko et al. [40]. The authors found out that that the formation rates of singlet oxygen ($^1O_2$) radical, hydroxyl radicals ($^{\bullet}OH$) and carbonate radical and their steady state concentration

decreased along the estuarine salinity gradient as a consequence of a decrease of terrestrial humic-like DOM. Parker et al. [41] focused their study on the on the triplet state excited natural organic matter (^{3}NOM*). The authors observed that the steady-state concentration of excited triplet state of ^{3}NOM* increased with an increase in salinity, regardless of the salt used, due to a decline of the ^{3}NOM* rate decay constant. The enhanced photodegradation rate constant of chlorpyrifos in saline waters enriched with SRNOM at higher concentrations (20 mgL^{-1}) could be a consequence of changes in the ^{3}NOM* lifetime which increases the rate of indirect photolysis of the target compound. Another possibility is that ^{3}NOM* reacts with chloride, or other halide ions, to produce the oxidizing $Cl_2^{\bullet-}$ (or $X_2^{\bullet-}$) radical anion [42]. Formation of such species from reaction of halide ions with triplet states has previously been reported [43].

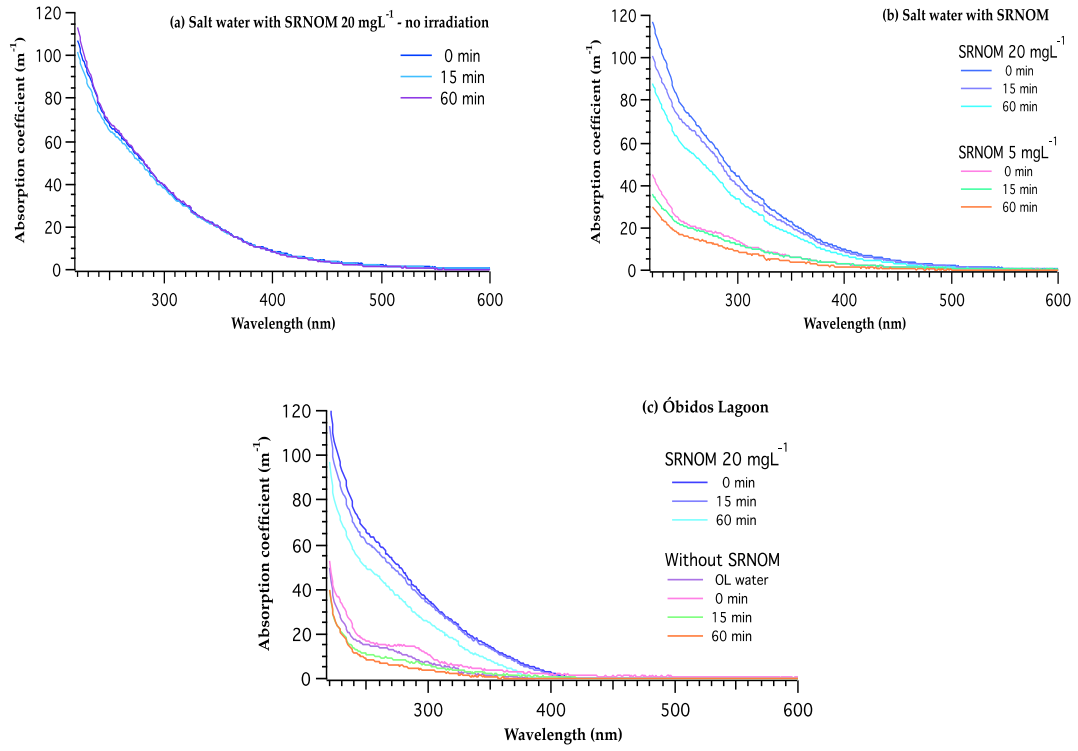

Fig. 6.2 – Absorbance spectra of: (**a**) salt water (34.6 gL^{-1}NaCl) enriched with SRNOM at 20 mgL^{-1} without irradiation; (**b**) salt water (34.6 gL^{-1}NaCl) enriched with SRNOM at 20 mgL^{-1} and 5 mgL^{-1} after 0, 15 and 60 min of irradiation; (**c**) natural saline water (Óbidos Lagoon) with and without SRNOM (20 mgL^{-1}) after 0, 15 and 60 min of irradiation. OL water is the spectrum of the water from Óbidos Lagoon measured before any spiking of chlorpyrifos and SRNOM. At the initial time chlorpyrifos was at 1 mgL^{-1}.

Table 6.4 – Spectral slopes (S) of the different types of waters enriched with SRNOM at 5 or 20 mgL^{-1}. The coefficient of correlation (r^2) is under brackets.

	$S_{275-295}$ (nm^{-1})		
Water	**0 min**	**15 min**	**60 min**
Deionized water + SRNOM (20 mgL^{-1})	0.013 (0.99)	0.013 (0.99)	0.014 (0.99)
Salt water + SRNOM (20 mgL^{-1})	0.013 (0.99)	0.015 (0.99)	0.015 (0.99)
Salt water + SRNOM (5 mgL^{-1})	0.010 (0.90)	0.013 (0.98)	0.016 (0.96)
Natural saline water	0.039 (0.79)	0.013 (0.86)	0.019 (0.82)
Natural saline water + SRNOM (20 mgL^{-1})	0.014 (0.99)	0.015 (0.99)	0.017 (0.99)
Natural saline water**	0.023 (0.99)	-	-
Salt water + SRNOM (20 mgL^{-1}, no irradiation)	0.013 (0.99)	0.013 (0.99)	0.013 (0.99)

** – Natural saline water without addition of chlorpyrifos.

Several studies of chlorpyrifos photolysis have been carried out under different conditions of pH, concentrations, types of formulation, in closed and open systems mainly with xenon lamps as summarized in Table 6.5 [13, 44-46]. Photodegradation rate constants obtained by the different studies were lower than the ones found herein with the MP Hg lamp. The corresponding half-lives are in the range of the 13.3 min to 13.9 days and were shorter in open systems than in closed systems. Differences in the photodegradation rates can be due to the type of lamp that was used as well as the different conditions applied. Xenon lamps are very high intensity visible light emitters with some large peaks extending into the near infrared range [47]. Their intensity drops significantly below 300 nm which decreases their effectiveness towards chlorpyrifos degradation [47]. MP Hg lamps are characterized by their strong polychromatic output in the range of 200 to 400 nm [21, 47]. This overlaps the two maximum absorbance peaks of chlorpyrifos (Fig. 6.3). MP Hg lamps are, therefore, more efficient towards chlorpyrifos degradation as confirmed by the results presented herein. The compound has no significant absorbance at 254 nm making LP Hg lamps less effective than xenon lamps [48].

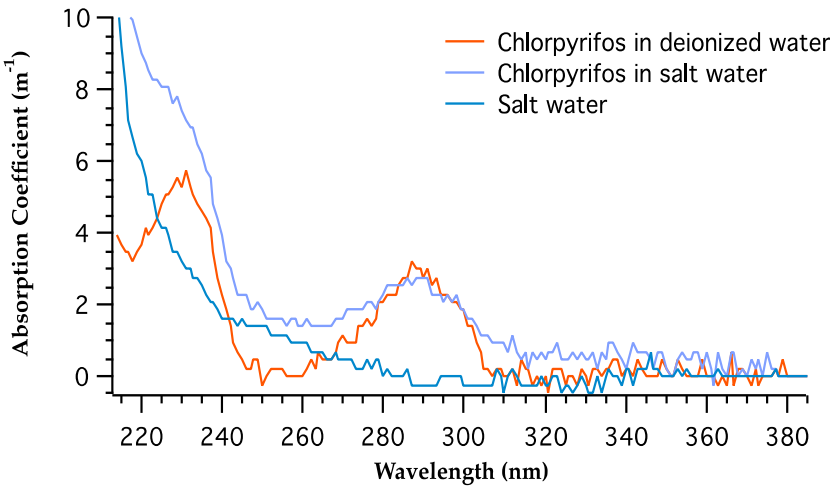

Fig. 6.3 – Absorbance spectra of chlorpyrifos (1 mgL^{-1}) dissolved in unbuffered deionized water and in salt water (34.6 gL^{-1} NaCl), and only salt water (34.6 gL^{-1} NaCl) as blank in the range of 214 nm to 395 nm.

Table 6.5 – Summary of photodegradation rate constants and/or half-lives of chlorpyrifos under different conditions [13, 44-46, 48].

UV Lamp	Rates	Half-life	Concentration	Medium	System	Reference
Xenon arc (solar simulator)	0.032 – 0.053 day^{-1}	7.8 – 13.9 days	0.3 mgL^{-1}	Buffered distilled water (pH 5.0, 6.9 and 8.0)	Closed system	[13]
LP Hg lamp and natural sunlight	--	44 to 58 min	72 mg (1 mL *of evaporated standard solution prepared in acetone*)	3 different types of formulations (Dursban, Bestban and Teragard)	Closed system	[48]
Xenon arc (solar simulator)	0.0115 – 0.01160 min^{-1}	59.9 – 60.1 min	29 – 95 μgL^{-1}	Unbuffered distilled water and natural water	Closed system	[46]
Xenon arc (solar simulator)	--	5% remained after 60 min of irradiation	100 mgL^{-1}	Unbuffered distilled water (methanolic solution)	Closed system	[45]
Xenon parabolic	0.052 min^{-1}	13.3 min	7 mgL^{-1}	Unbuffered deionized water	Open and agitated medium	[44]

6.3.2 Study of hydrolysis

Chlorpyrifos was stable towards hydrolysis in unbuffered deionized water and in saline waters. The loss ranged from 2 to 6% over the measured time. These results are in agreement with published data. Reported hydrolysis half-lives of chlorpyrifos in aqueous systems, including saline waters, range from a few hours up to 210 days at pH between 5 to 9 [8, 15].

6.3.3 Volatilization

The volatilization of chlorpyrifos (Fig. 6.4) also followed pseudo-first-order kinetics and resulted in a significant loss of chlorpyrifos in both deionized and saline waters and in the saline water enriched with SRNOM (Table 6.6). Volatilization was more rapid in saline water than in deionized water under aerated conditions. In non-aerated waters, half-lives of chlorpyrifos were lower in the range of 86 to 99 min. Aeration reduced the half-life of chlorpyrifos by 70% in deionized water and 90% in saline waters. Addition of SRNOM to aerated salt waters decreased the volatilization rate constant by 35%. Aeration increases the turbulence the bulk phase and hence the rate of mass transfer of chlorpyrifos at the gas-liquid interface [49, 50]. The air introduced into the liquid phase breaks the equilibrium between liquid and gas phase and results in the removal of compounds [49]. The salinity decreases the solubility of the compound increasing its tendency to escape to the gas phase [51]. Similar findings were reported by C. Thomas et al. [12] who studied the dissipation of 1 mgL^{-1} of chlorpyrifos from tap, river and brackish (13‰) waters. The authors found out that dissipation of chlorpyrifos was significantly greater for aerated natural waters than for non-aerated waters and the difference was more pronounced for brackish water. In both conditions (aerated and non-aerated), C. Thomas et al. [12] found higher half-lives in the range of the 3.2 hours to 16.8 days. Under non-aerated conditions, the half-lives were 16.8 days, 9.66 days and 2.5 days for tap, brackish and river water, respectively. The discrepancies

between the half-lives found by C. Thomas et al. [12] and the present work can be explained by the differences in the experimental conditions. In the work of C. Thomas et al. [12], the authors used a commercial formulation of the insecticide. Pesticide commercial formulations usually contain emulsifiers and other inert compounds that increase the solubility and the stability of the active substances reducing their volatilization even with good mixing [2, 48, 52, 53]. The authors also worked at a lower airflow rate (1.2 $Lmin^{-1}$) using a higher volume of water (3000 mL), which might have a significant impact on rate of mass transfer and consequently on the volatilization of chlorpyrifos. Results show that the addition of SRNOM to salt waters decreased chlorpyrifos volatilization probably due an increase of its apparent solubility. Enhancement of solubility is most likely due to complexation or interactions of chlorpyrifos with the dissolved organic matter [54]. Huang et al. [20] observed a strong affinity of DOM, especially humic acids for chlorpyrifos which reduced its sorption by soil. The authors found a positive and linear correlation between the apparent solubility of the compound and the concentration of DOM. The effectiveness of DOM in enhancing solute solubility appears to be largely controlled by DOM source, molecular size and polarity [20, 54].

Chlorpyrifos is an organophosphate pesticide with a moderate vapor pressure (Table 6.2) and an intermediate Henry´s Law constant in the range of 0.3 - 1.8 Pa m^3mol^{-1} [1, 8]. According to Lyman et al. [55] for compounds with Henry´s constant (H) between 10^{-1} and 1 Pa m^3mol^{-1} the rate of volatilization is controlled by the slow rate of diffusion through the air. Volatilization is slow but possible. The rate at which chlorpyrifos volatilizes in the environment depends on its physical-chemical properties, especially vapor pressure and Henry´s Law constant and also on the properties of the environmental matrix in which it is released. Results show that the salinity, the aeration as well as the dissolved organic matter can have a significant effect on the dissipation of the compound from aquatic ecosystems. While photolysis of chlorpyrifos is most

likely to be the main transformation process in freshwaters, both volatilization and photolysis could be important pathways of chlorpyrifos dissipation in aerated coastal waters.

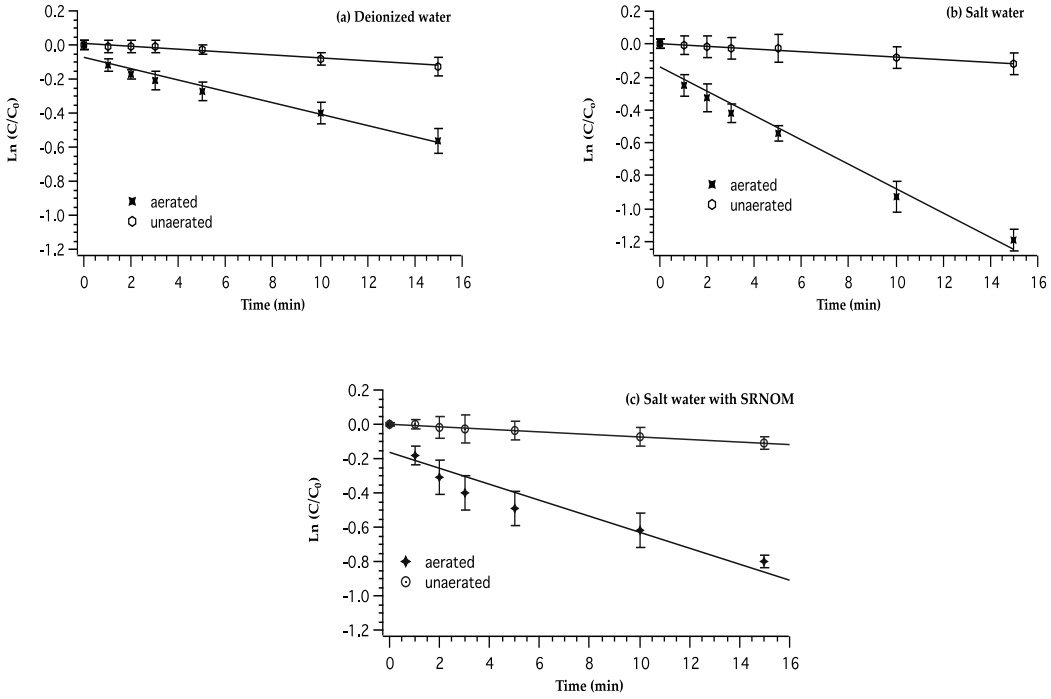

Fig. 6.4 – Dissipation kinetics of chlorpyrifos under aerated (airflow 2 $Lmin^{-1}$) and unaerated conditions in: (**a**) unbuffered deionized water; (**b**) salt water (34.6 gL^{-1} NaCl) and (**c**) salt water enriched with SRNOM at 20 mgL^{-1}.

Table 6.6 – Volatilization rate constants of chlorpyrifos in unbuffered deionized, salt water (34.6 gL^{-1}) and salt water with SRNOM (20 mgL^{-1}). Values are expressed and as mean values ± standard deviation (n = 3).

Water	Unaerated k (min^{-1})	$t_{1/2}$ (min)	Aerated k (min^{-1})	$t_{1/2}$ (min)
Deionized	0.008 ± 0.002 (r^2 = 0.98)	86	0.027 ± 0.003 (r^2 = 0.97)	26
Salt	0.007 ± 0.004 (r^2 = 0.96)	99	0.074 ± 0.002 (r^2 = 0.96)	9
Salt water with SRNOM (20 mgL^{-1})	0.007 ± 0.004 (r^2 = 0.95)	99	0.048 ± 0.01 (r^2 = 0.94)	14

6.3.4 Photodegradation products

The main degradation product of chlorpyrifos photolysis in unbuffered deionized water and saline waters was 3,5,6-trichloro-2-pyridinol (Annex C, Fig. C.1 and Fig. C.2). The oxon analogue obtained by oxidation of the P = S bond of chlorpyrifos to P = O was not detected [17, 46, 56]. The oxon is a very unstable compound that tends to hydrolyze more rapidly than chlorpyrifos especially at high pH [17]. Addition of SRNOM did not change the products of chlorpyrifos degradation, and TCP was also found to be the main degradation product in all other published studies [2, 7]. This indicates that the mechanism of chlorpyrifos degradation in saline waters is similar to that in distilled and natural freshwaters. In addition, the medium-pressure Hg lamp produced the same degradation product as other light sources, although variations occur in the photodegradation rate of the parent compound [45]. It is likely that TCP, like trichlorophenols, undergoes further photodegradation under environmental conditions [57, 58]. However, this will be the subject of future studies.

6.4. Conclusions

The photolysis reactions of aqueous chlorpyrifos solutions have been studied in saline and deionized water. It was shown that they are faster when MP Hg lamps are used when compared with other irradiation sources, such as xenon or low-pressure mercury lamps. Also, an enhancement in rate was observed in salt water with high SRNOM concentrations. Although chlorpyrifos has a tendency to be directly degraded by UV radiation, dissolved organic matter such as present in estuarine and lagoon waters favours the increase of indirect photodegradation rate. In low-organic matter waters, such as oceanic waters, and under aerated conditions, volatilization can be an important pathway together with photolysis and to a less extent hydrolysis.

Photodegradation and volatilization of pesticides, and therefore their fate in the aquatic environment need to account for the influence of salinity, water aeration in conjunction with the amount and type of dissolved organic matter.

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7

A New 2D Separation for Characterizing Dissolved Organic Matter

Highlights

- The new SPE-based methodology enable fractionation of DOM based on its hydrophobicity
- Fractions show a significant compositional changes with the increasing of eluent hydrophobicity
- Fractions show RP-HPLC profiles that differentiate fresh from marine waters
- The improved separation is applicable to DOM from different sources

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Abstract

A new method of fractionation of dissolved organic matter (DOM) was developed based on a three-step gradient elution solid phase extraction (SPE). DOM was isolated by SPE and fractionated sequentially by elution with a mixture of methanol:water (PPL I), methanol:acetonitrile:water (PPL II) and methanol:acetonitrile (PPL III). The method was applied to marine and freshwaters. The corresponding fractionated DOM extracts were characterized by UV-Vis, EEMs, ^{1}H NMR and FT-IR spectroscopy and RP-HPLC with diode array detection. DOM recoveries of 60 and 78% were achieved with PPL cartridges. Results showed a significant compositional change in the PPL extracts with an increase of eluent hydrophobicity. The intermediated hydrophobic fraction (PPL II) presented the lowest values of the spectra slope ratio S_R ($S_{275-295}/S_{350-400}$) suggesting that the PPL fraction II is richer in DOM with high molecular weight. The total fluorescence increased from the more polar fraction (PPL I) to the intermediated polar fraction PPL II and decreased in the less hydrophilic PPL III fraction. This trend was in general positively correlated with the molecular weight or size. ^{1}H NMR data show a decrease of the %carbohydrates and an increase of the percentage of material derived from linear terpenoids (MDTL) with an increase of eluent hydrophobicity. The percentage of carboxylic-rich alicyclic molecules (CRAM) was higher in the intermediate hydrophobic extracts (PPL II) for marine waters with a low input of terrestrial DOM. FT-IR spectra showed the presence of highly conjugated aliphatic structures in all PPL fractions derived from the wetland waters. The improved RP-HPLC confirmed the selective fractionation of the DOM based on the polarity. Characteristic chromatographic profiles were obtained for the different type of waters.

Keywords: Dissolved organic matter; Solid phase extraction; Hydrophobicity; Allochthonous; Autochthonous; RP-HPLC.

7.1. Introduction

Estuaries and coastal lagoons are the interface where terrestrial and marine constituents meet, namely organic matter making them an important component of the global carbon cycle [1, 2]. The flux of dissolved organic carbon from rivers to estuaries and oceanic margins is estimated to be from 0.22 to 0.40 Gt-C yr^{-1} (Gt = 10^{15} g) in the same order of magnitude as the annual production of semi-labile DOM in open ocean (1.2 Gt-C yr^{-1}) [3, 4]. This organic input is likely to influence markedly the carbon cycle, the optical characteristics of the water, its quality as well as the fate of the natural and man-made compounds [2, 3, 5]. Lagoons and estuaries are highly impacted by urbanization, agricultural runoff, and riverine anthropogenic contaminants. The predominance of allochthonous *vs.* autochthonous DOM in lagoons and estuaries depends on many factors [3]. Terrestrial or allochthonous organic matter are believed to contribute to phytoplankton dynamics and consequently to coastal environment biodiversity [6, 7]. Because of the restricted exchange with the ocean, coastal lagoons tend to have low flushing rates increasing primary productivity [8]. Estuaries can have a different hydrology and the turbulent mixing of fresh and salt water generates abrupt changes in temperature, salinity, pH and consequently on DOM type, its concentration and fate [9-11].

Wetlands also play an important role in the global carbon cycle. Approximately 15% of the global terrestrial carbon flux from rivers to coastal environments is estimated to be from wetlands, although this type of ecosystem covers only 5 – 8% of the earth´s land surface [12]. Wetlands are also used in wastewater treatment [13, 14]. The nature of DOM in wetlands receiving treated effluents is thought to depend on the developmental stage of the wetland and the vegetation patterns [13]. Understanding the relative contributions of allochthonous and autochthonous DOM to estuaries and wetlands is an important aspect of monitoring the viability of these environmentally sensitive preserves. The rate of production and degradation

of DOM in wetlands or any aquatic system is dependent on the biological and physic-chemical conditions of the medium as well as on DOM chemical structure [6, 13, 15-17]. Knowledge of DOM molecular-level composition is thus crucial for a better understand and prediction of its sources, behaviour, bioavailability and impact on the global carbon cycle. In some ecosystems like the constructed wetlands DOM chemical characterization can improve wastewater treatment efficiency.

The characterization of unfractionated source and estuary waters includes UV-visible [18] and three-dimensional excitation emission fluorescence spectroscopy (EEMs) [19-25]. Fractionation is essential because DOM, composed of thousands compounds, is one of the most complex naturally occurring mixtures [26, 27]. The low concentration of DOM in natural waters, ranging from 0.1 – 1 mgL^{-1} in marine waters to 20 – 50 mgL^{-1} in riverine waters, presents a special challenge for its extraction and isolation [28]. The high salt concentration in marine waters (35 gL^{-1}) requires a technique that also desalts the DOM. Fractionation of DOM according to polarity has been carried out by sequential sorption of water samples at a specific pH on acrylic ester resins (XAD-8) and styrene-divinylbenzene resins (XAD-4) [29, 30]. Elution is usually done with a mixture of acetonitrile and water (3:1) [29, 31]. This traditional extraction approach is time consuming and became less applied since XAD resins are no longer manufactured [32]. More recently, solid phase extraction (SPE) and elution with methanol or acetonitrile are used with either a C-18 [30, 33] or a modified polymeric styrene-divinylbenzene adsorbent [31, 34-36]. Reversed-phase high performance liquid chromatography (RP-HPLC) of SPE extracted DOM has been used to characterize changes in DOM from riverine to estuary waters and marine waters [37-42]. Characterization techniques include nuclear magnetic resonance spectroscopy (NMR), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), Fourier transform infrared spectroscopy (FT-IR), EEMs, and UV-Visible spectroscopy [32, 40, 43, 44].

This study presents a developed three main step gradient SPE elution scheme to fractionate the DOM and improved an RP-HPLC gradient methodology to characterize the SPE-based fractions. The fractionated DOM was also characterized by UV-Visible spectroscopy, EEMs, ^{1}H NMR and FT-IR spectroscopy. The results showed that these new fractionation and separation methods, based on eluent hydrophobicity, allow both the inter- and intra- sample comparison of the lagoon, estuary and wetland waters. These procedures are applicable to DOM in general and the improved separation will enhance on-line analytical techniques.

7.2. Materials and methods

7.2.1 Reagents

Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (ACS reagent) and water (Optima™, LC/MS grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Whatman GF/F glass microfiber filters (0.7 µm nominal pore-size) and sodium deuterium oxide (NaOD) 30% (wt) were purchased from Sigma-Aldrich (St. Louis, MS, USA). The 5 mm NMR tubes (ref. 507-HP-7) were obtained from Norell (Landisville, USA). Bond Elut PPL cartridges were obtained from Agilent Technologies (Santa Clara, CA). Suwannee River natural organic matter (SRNOM, 1R101R) was purchased from the International Humic Substances Society (IHSS, St. Paul MN, USA). Deuterium oxide (D_2O) was obtained from EURISO-TOP (Saint-Aubin, France).

7.2.2 Sampling

Water samples (1 to 2 L) were collected in three aquatic systems: three sites in Óbidos Lagoon, a costal lagoon located in Western coast of Portugal (Óbidos Lagoon, Annex, D, Fig. D.1), three sites in the Newport Back Bay (NPBB, Annex D, Fig. D.2), (Newport Beach, CA, USA) and two sites in the

San Joaquin wetlands (IRWD, Annex D, Fig. D.2) (Irvine Ranch Water District (IRWD) Irvine, CA USA). After collection the water samples were filtered through pre-combusted Whatman GF/F glass microfiber filters (0.7 µm nominal pore-size). Samples were acidified with glacial acetic acid to a final concentration of 2% v/v (final pH 2.5 – 3.0) and kept at 4 ^{o}C for DOM extraction within 48 h. A more detailed description of the sampling sites and sample characteristics can be found in supplemental information (Annex D, Table D.1).

7.2.3 DOM extraction

Extraction of DOM from water samples was carried out by solid phase extraction (SPE) using Bond Elut PPL cartridges. The PPL cartridges were conditioned according to the manufacturer´s instructions using two column volumes of methanol followed by LC-MS water and LC-MS water at 2% of acetic acid. The acidified water (1 L) was loaded on to the cartridge bed under vacuum. The cartridges were washed with LC-MS water to remove salts before air-drying. The bound DOM was eluted sequentially with a mixture of eluents as illustrated in Fig. 7.1. Three main extracts were obtained: PPL I (methanol:water, 1:1, v:v), PPL II (methanol:acetonitrile:water, 1:1:2, v:v:v) and PPL III (methanol:acetonitrile, 1:1, v:v). To confirm that all DOM was extracted an elution was carried out with methanol. The same extraction procedure was used to fractionate SRNOM (50 mgL^{-1}). The PPL extracts were dried under vacuum (Savant Speedvac®, ThermoScientific, USA) and re-dissolved in 5 ml of LC-MS grade water (Fisher Sci., Optima™) for spectroscopic analysis. The UV-visible and EEMs spectra of the replicate PPL fractions from the Óbidos Lagoon (OL) were analysed separately before averaging. The OL replicates were combined for NMR and FT-IR analysis.

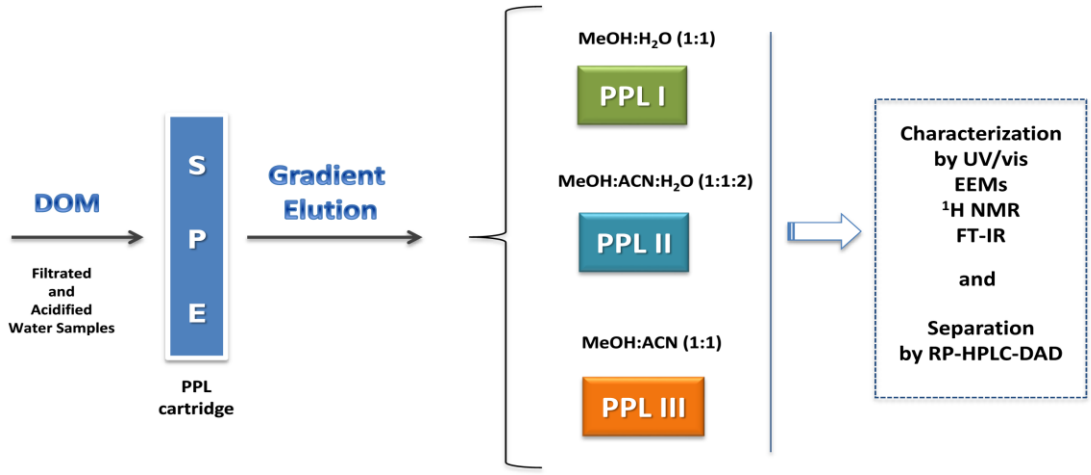

Fig. 7.1 – Scheme of the developed PPL fractionation of DOM. A mixture of methanol, water and acetonitrile was used to elute DOM from the PPL cartridges in a stepwise gradient mode. The obtained PPL fractions were separated by RP-HPLC and characterized using different spectroscopic approaches.

7.2.4 Reversed-Phase High Performance Liquid Chromatography

Lagoon, estuary, and wetland waters and their respective PPL extracts were analysed by reversed phase high performance liquid chromatography (RP-HPLC) using an Agilent 1200 (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a 100 µL loop injector, a diode array detector (DAD, G1315C). DOM PPL fractions were separated in a Zorbax Eclipse XDB-C18 column (4.6×150 mm, 5 µm, Agilent Technologies, Inc., Santa Clara, CA, USA) with an Agilent guard-column (Eclipse XDB 4.6×12.5 mm, 5 µm, Agilent Technologies, Inc., Santa Clara, CA, USA). Fractionation was performed using a gradient comprised of 0.2% acetic acid and an acetonitrile mobile phase. Two different gradients were used for PPL fractionation. Gradient 1 (2 – 12.5% acetonitrile, 25 min, followed by a 3-step gradient) was used to fractionate the 50% PPL fractions (Annex D, Table D.2). The more hydrophobic PPL fractions were separated using Gradient 2 (Annex D, Table D.3) with a steeper initial gradient (2 – 12.5% acetonitrile, 5 min). More detailed information about the gradient programs can be found in the supplementary material (Annex D, Tables D.2 and D.3).

7.2.5 UV-Vis Spectroscopy

The UV-Visible spectra were measured on a Cary 100 (Agilent Technologies, Inc., Santa Clara, CA, USA) dual-beam spectrophotometer using 1 cm path length quartz cuvettes. Samples were scanned in the range of 200 to 800 nm. To minimize temperature effects samples were equilibrated to room temperature. Bulk filtered water was analysed without any dilution. The re-dissolved PPL extracts were diluted from 1:10 to 1:1000 depending on the source waters. The absorption coefficient α (m^{-1}), at wavelength λ, was calculated using equation 1:

$$\alpha = 2.303 \times A(\lambda)/l \qquad (1)$$

where $A(\lambda)$ is the absorbance measured across the cell path length l (m). To quantify the absorption variations a spectral slope ratio was calculated ($S_R = S_{275\text{-}295}/S_{350\text{-}400}$) as well as the absorbance ratio E2:E3 ($250_{nm}/365_{nm}$) [18, 45].

7.2.6 Excitation Emission Matrix fluorescence Spectroscopy

Excitation-emission matrix spectra (EEMs) of the whole water samples and the re-dissolved PPL extracts were acquired using a Fluoromax-4 spectrometer (Horiba Jobin Yvon, Inc.). Fluorescence spectra were measured in the range of 240 to 600 nm at 5 nm intervals (excitation: 240 – 500 nm; emission: 290 – 600 nm). Spectra were corrected for Raman scattering and calibrated to quinine sulphate units (QSU) using FL Toolbox. The DOM was characterized by the reference excitation/emission: peak A (Ex/Em = 260/380-460 nm; humic-like), peak C (Ex/Em = 350/420-480 nm; humic-like), peak M (Ex/Em = 312/380-420 nm; marine like) and peak T (Ex/Em = 275/340 nm; tryptophan/tyrosine-protein-like material) [25, 46]. The fluorescence of four peak areas (QSU) was calculated as a percentage of the total fluorescence.

7.2.7 ^{1}H NMR and FT-IR analysis

Freeze-dried samples were re-dissolved in D_2O and adjusted to pH 8 with NaOD and transferred to 5 mm NMR tubes. Samples from sites A, B and C of Newport Back Bay were concentrated in only one sample. The ^{1}H NMR, spectra were acquired in a Bruker AVANCE III 400 spectrometer operating at a Proton frequency of 400.15 MHz (^{1}H). A QNP 5 mm probe head was used and the temperature was set to 298 K in all experiments. The proton chemical shift was calibrated relative to acetic acid (internal reference, δ = 4.9207 ppm). Experimental conditions for the acquisition of ^{1}H NMR - first proton program to select frequency offset, followed by ZGESGP program acquisition with spectral window of 16 ppm and 1024 transients. ^{1}H NMR spectra were integrated using MestReNova v. 9.1.0 (Mestrelab Research S.L.). Integrated values were expressed as percentage of total peak area [46]. After NMR analyses LC-MS water grade was added to the extracts that were afterwards lyophilized for FT-IR analyses. Infrared spectra were recorded on the freeze-dried samples with a Thermo Scientific Nicolet 6700 FT-IR spectrometer (Thermo Electron Corporation, Madison, USA) equipped with a Smart iTR attenuated total reflectance (ATR) sampling accessory with a single reflection diamond crystal. The FT-IR spectra were averaged from 32 scans and recorded over the range of 4500 - 525 cm^{-1} at a resolution of 4 cm^{-1}.

7.3. Results and discussion

7.3.1 Optical properties of the sampled waters and respective PPL fractions

7.3.1.1 UV-Vis spectra

The UV-Visible spectra for the Óbidos Lagoon (OL, Fig. 7.2a) are typical of coastal/marine DOM [18] and reflects the high saline content [47]. The spectra for the salt-free OL PPL extracts (Fig. 7.2b) have a profile more like DOM in general [48]. The A_{250nm} (Table 7.1) is an indicator of DOM

concentration and increases by six-fold from OL site 1 to site 3 where sites 2 and 3 have high terrestrial inputs of DOM [49, 50].

The spectra slopes ($S_{275\text{-}295\ nm}$ and $S_{350\text{-}400\ nm}$), the spectral slope ratio (S_R,) and *E2:E3*, inversely correlated with molecular weight [18, 51], are summarized in Table 7.1. For the bulk waters the $S_{275\text{-}295}$ values ranged from 0.015 nm^{-1} to 0.023 nm^{-1} where the lower values are typical of terrestrial DOM (SRNOM = 0.013 nm^{-1}) while the higher values indicate a more autochthonous influence [18, 45, 48, 52]. The spectral slope ($S_R = S_{275\text{-}295}/S_{350\text{-}400}$) increase from terrestrial (0.71), to estuary (1.1) to ocean waters (9.4 in the Sargasso Sea) [18]. In this study S_R = 0.81 for SRNOM is representative of freshwater DOM. The S_R values for the OL water decrease from the ocean inlet (2.05), to site 2 (1.48), and to site 3 (1.16) reflecting the increase in terrestrial DOM and molecular weight.

S_R values for the OL PPL fractions show the same spatial trend from high (site 1) to low (site 3) but the absolute values are less than the bulk water. The ratio *E2:E3* shows the same negative correlation with molecular size. The *E2:E3* values are higher in all PPL fractions indicating a decrease molecular size perhaps due to fewer intermolecular interactions [53]. The DOM concentration for the NPBB sites is similar at all three sites ($A_{250} \approx 4\ m^{-1}$) and the high values for $S_{275\text{-}295}$ (0.022 – 0.023 nm^{-1}) are indicative marine DOM. The slope ratios were not calculated for the bulk water because of the low correlation coefficients (r^2) values associated with the low DOM concentration. The values of $S_{275\text{-}295}$ for the PPL fractions are lower than the bulk water. The DOM concentration in the IRWD outflow wetland water (10.4 m^{-1}) is approximately 20% higher than the inlet water (8.4 m^{-1}). The outflow $S_{275\text{-}295}$ (0.017 nm^{-1}), *E2:E3* (6.32), and S_R (0.94) are higher than the inflow indicating an increase in lower molecular weight through the wetland, perhaps due to DOM photodegradation [18]. The outflow fractions from PPL I and PPL III have higher *E2:E3* and S_R values suggesting that they are more photo-reactive than PPL II. While the relative proportions of DOM are the

same in PPL I and II, the DOM in the more hydrophobic outflow PPL III fraction is almost double that of the inlet. DOM concentration for the standard SRNOM was higher in PPL II (96.7 m^{-1}). In general the PPL II fractions presented the lowest values of S_R suggesting that the intermediated hydrophobic fraction is richer in DOM with high molecular weight.

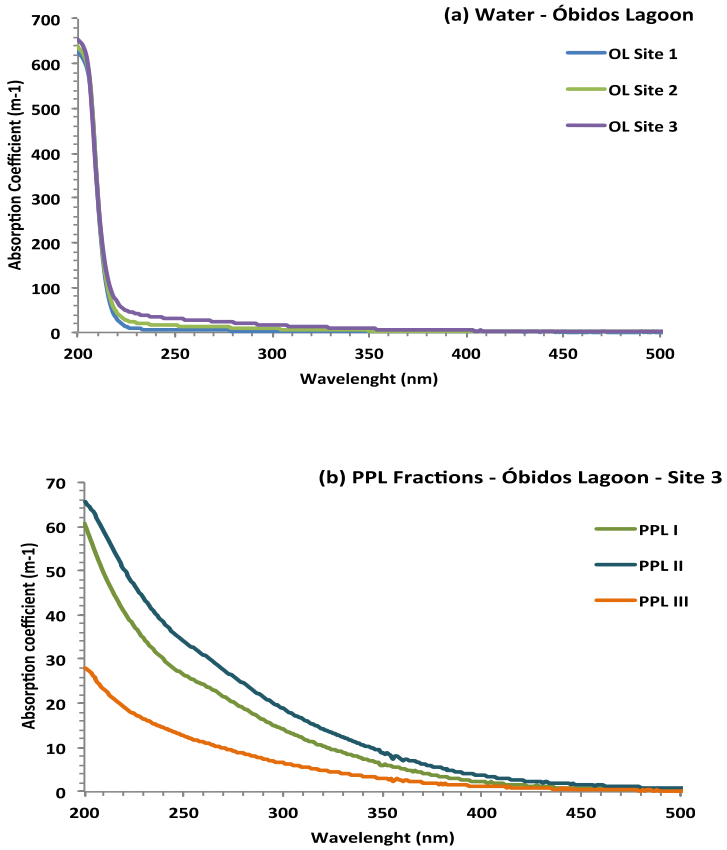

Fig. 7.2 – UV-Visible spectra of (**a**) water sample collected on the site 3 of Óbidos Lagoon and (**b**) PPL fractions (PPL I, PPL II, PPL III) of water sample collected on site 3 of Óbidos Lagoon.

Table 7.1 – Spectra slopes (S, nm^{-1}), spectral ratio (S_R = $S_{275-295}/S_{350-400}$) and E2:E3 (A_{250}/A_{365}) of the studied natural waters and the standard Suwannee River natural organic matter (SRNOM) and corresponding PPL fractions.

Bulk water	$S_{275-295}$ (nm^{-1})	r^2	$S_{350-400}$ (nm^{-1})	r^2	S_R	*E2:E3*	A_{250} (nm^{-1})
OL Site 1	0.017	0.99	0.008	0.70	2.05	5.10	5.50
OL Site 2	0.016	0.99	0.011	0.92	1.48	4.56	15.3
OL Site 3	0.015	0.99	0.013	0.99	1.16	4.56	30.6
NPBB Site A	0.023	0.99	nd	nd	nd	nd	4.10
NPBB Site B	0.022	0.99	nd	nd	nd	nd	4.70
NPBB Site C	0.023	0.98	nd	nd	nd	nd	4.40
IRWD Inlet	0.016	0.99	0.018	0.91	0.89	6.05	8.40
IRWD Outflow	0.017	0.99	0.018	0.91	0.94	6.32	10.4
SRNOM (50 mgL^{-1})	0.013	0.99	0.016	0.99	0.81	4.04	77.2
PPL I							
OL Site 1	0.018	0.99	0.013	0.98	1.38	5.76	2.70
OL Site 2	0.015	0.99	0.019	0.95	0.78	6.07	10.7
OL Site 3	0.015	0.99	0.020	0.99	0.72	5.70	26.3
NPBB Site A	0.018	0.98	0.018	0.62	1.03	7.93	4.00
NPBB Site B	0.017	0.99	0.020	0.99	0.92	5.76	5.20
NPBB Site C	0.017	0.97	0.015	0.98	1.13	5.60	4.80
IRWD Inlet	0.015	0.99	0.019	0.99	0.82	6.11	51.6
IRWD Outflow	0.017	0.99	0.019	0.99	0.87	6.69	50.6
SRNOM	0.015	0.99	0.023	0.99	0.66	4.64	66.3
PPL II							
OL Site 1	0.018	0.98	0.016	0.98	1.13	6.16	3.40
OL Site 2	0.013	0.98	0.018	0.96	0.74	5.15	12.1
OL Site 3	0.013	0.99	0.018	0.99	0.75	4.98	34.1
NPBB Site A	0.014	0.98	0.016	0.77	0.87	4.48	5.10
NPBB Site B	0.014	0.99	0.011	0.99	1.26	4.17	4.80
NPBB Site C	0.016	0.99	0.014	0.99	1.14	5.14	4.10
IRWD Inlet	0.013	0.99	0.016	0.99	0.81	5.02	47.6
IRWD Outflow	0.015	0.99	0.017	0.99	0.82	5.65	47.0
SRNOM	0.013	0.99	0.020	0.99	0.65	5.16	96.7
PPL III							
OL Site 1	0.019	0.96	0.015	0.95	1.26	6.63	2.01
OL Site 2	0.016	0.98	0.016	0.76	0.99	6.07	5.40
OL Site 3	0.014	0.99	0.017	0.93	0.82	5.22	12.7
NPBB Site A	0.015	0.88	0.028	0.24	0.52	7.27	1.70
NPBB Site B	0.017	0.97	0.011	0.92	1.56	5.57	1.90
NPBB Site C	0.016	0.80	0.010	0.66	1.60	4.16	1.41
IRWD Inlet	0.015	0.99	0.016	0.98	0.94	6.16	20.7
IRWD Outflow	0.015	0.99	0.016	0.98	1.14	6.92	38.7
SRNOM	0.013	0.99	0.017	0.97	0.76	4.64	21.6

Note: r^2 – linear correlation coefficient; nd – not detected; OL = Óbidos Lagoon (average of three samples); NPBB = Newport Back Bay; Irvine Ranch Water District San Joaquin wetlands = IRWD.

7.3.1.2 EEMs

The EEMs fluorescence for peaks A, C, M, and T was calculated as a percentage of the total fluorescence (Table 7.2). The total fluorescence followed the same trends as the A_{250} increasing from OL site 1 to OL site 3. The DOM fluorescence of the NPBB water was 8% higher at site B (NPBB inlet) than at sites A and C. The IRWD outflow water was more fluorescent (+14%) than the inlet water. The recovery of DOM from the PPL fractionation of the IRWD wetland water was calculated from the total fluorescence of the bulk water and the PPL flow through (Table 7.2). There was also a difference in the fluorescence intensities of the four peaks. The inlet flow-through had highest in peak T fluorescence (31%) and the lowest peak A fluorescence (31%). Surprisingly, peak M fluorescence was highest in the outflow (33%) yet showed little variation in the PPL fractions.

Analysis of the individual peaks A, C, M, and T for the OL waters their PPL extracts show differences in fluorescence based on location and hydrophobicity. In the Óbidos Lagoon waters peak T fluorescence decreased (-79%) while peak A + C fluorescence increased (+24%) as the waters transitioned from a marine to a more terrestrial-like environment. Peak M was not significantly different at the three sites. Peak T in PPL I and II showed the same decrease with distance from the ocean. However, peak T was significantly higher in the PPL III fraction at OL sites 1 and 2 suggesting a different source for the fluorescence. Peaks A and C were the dominant peaks (Annex D, Fig. D.3). Peaks A + C fluorescence increased from PPL I to PPL fractions II and decreased in PPL III. This trend was positively correlated with molecular size [54]. In general the total fluorescence of the PPL fractions follow the trend: PPL II > PPL I > PPL III (Table 7.2).

Table 7.2 – EEMs integration results for the PPL fractions, natural waters, and SRNOM expressed as percentage of total area of the fluorescence (QSU).

	% Total EEMs Fluorescence				Total A+C+M+T Fluorescence (QSU)
Bulk water	**A**	**C**	**M**	**T**	
OL Site 1 (*no dilution*)	43	16	17	23	21
OL Site 2 (*no dilution*)	52	21	17	10	72
OL Site 3 (*no dilution*)	56	22	16	5	161
NPBB Site A (*no dilution*)	50	19	17	13	22
NPBB Site B (*no dilution*)	48	16	17	19	28
NPBB Site C (*no dilution*)	51	18	17	15	19
IRWD Inlet (*no dilution*)	54	20	18	9	220
IRWD Outflow (*no dilution*)	55	19	18	7	254
SRNOM	67	17	14	2	
PPL I					
OL Site 1 (*1:100 diln*)	40	17	19	23	16
OL Site 2 (*1:100 diln*)	51	22	18	9	64
OL Site 3 (*1:100 diln*)	67	16	15	2	130
NPBB Site A (*1:100 diln*)	53	20	16	11	19
NPBB Site B (*1:100 diln*)	51	21	17	11	22
NPBB Site C (*1:100 diln*)	59	22	18	12	19
IRWD Inlet (*1:1000 diln*)	54	20	16	10	32
IRWD Outflow (*1:1000 diln*)	54	21	18	7	25
SRNOM (1:500 diln)	60	24	13	3	85
PPL II					
OL Site 1 (*1:100 diln*)	53	20	16	10	19
OL Site 2 (*1:100 diln*)	56	22	16	7	81
OL Site 3 (*1:100 diln*)	70	15	14	2	203
NPBB Site A (*1:100 diln*)	55	20	16	10	26
NPBB Site B (*1:100 diln*)	56	19	16	9	26
NPBB Site C (*1:100 diln*)	56	19	16	9	26
IRWD Inlet (*1:1000 diln*)	56	20	17	7	32
IRWD Outflow (*1:1000 diln*)	57	20	17	6	28
SRNOM (*1:500 diln*)	60	23	14	3	102
PPL III					
OL Site 1 (*1:100 diln*)	53	19	16	12	10
OL Site 2 (*1:100 diln*)	52	17	16	15	43
OL Site 3 (*1:100 diln*)	70	14	13	4	89
NPBB Site A (*1:100 diln*)	49	15	15	20	12
NPBB Site B (*1:100 diln*)	53	14	15	18	14
NPBB Site C (*1:100 diln*)	50	15	15	20	16
IRWD Inlet (*1:1000 diln*)	56	15	17	12	27
IRWD Outflow (*1:1000 diln*)	58	15	17	10	25
SRNOM (*1:500 diln*)	57	20	15	7	27
PPL Flow through					
IRWD Inlet (*no dilution*)	31	20	18	31	85
IRWD Outflow (*no dilution*)	41	25	33	10	61

Note: OL = Óbidos Lagoon; NPBB = Newport Back Bay; Irvine Ranch Water District San Joaquin wetlands = IRWD; Suwannee River natural organic matter = SRNOM. Bulk water and PPL Flow through was not diluted.

Peak T fluorescence for the NPBB PPL I and II fractions were approx. 10% of the total fluorescence. The fluorescence increased in PPL III to 20% at all three sites. The fluorescence of Peaks A + C was similar at all three sites but was lowest in PPL III. The peak T fluorescence was lowest in the IRWD wetland waters (≈ 10% of total) but was also enriched in PPL III. The peak A

fluorescence increased from PPL I to PPL III while peak C was the same (20%) for both the inlet and outflow. However, peak C was lowest in PPL III and corresponded with an increase in peak T. The PPL III fraction of SRNOM also shown an increase of the peak T fluorescence with a decrease of peak A and C fluorescence. T-peak can be derived from proteinaceous materials but also from other classes of organic materials with similar fluorescence characteristics [55]. Residual phenolic groups in their humic-like diagenetic products are considered to contribute to T-peak fluorescence [55]. The signal from the T peak can also be due to protein encapsulation as DOM ages [54].

7.3.2 Functional group: Characterization using ^{1}H NMR and FT-IR

7.3.2.1 ^{1}H NMR spectroscopy

Fig. 7.2 shows the ^{1}H NMR spectra of the PPL fractions of the water collected in site 3 of Óbidos Lagoon. Lagoon, estuary, and wetland spectra displayed the characteristic DOM chemical shifts regions [56-58] but were more highly resolved than the comparable regions in the SRNOM spectrum (Annex D, Fig. D.4 to D.8). The regions were compared in terms of the percentage of total peak area (%area) of the integrated proton resonances (Table 7.3) [59]. The regions are defined as: aromatic (8.9 – 6.9 ppm), anomeric carbohydrate carbon/conjugated unsaturated aliphatics (6.9 – 5.0 ppm) and methylene/ methane in carbohydrates or protein (5.0 – 3.4 ppm), CRAM (carboxylic-rich alicyclic molecules 3.4 – 1.9 ppm) [60], MDLT (material derived from linear terpenoids 1.9 – 1.0 ppm) [58], and intra aliphatic chains (1.0 – 0.6 ppm).

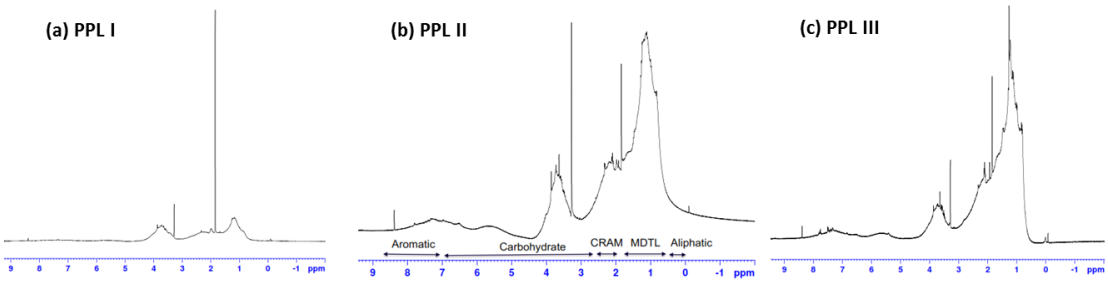

Fig. 7.3 – ^{1}H NMR spectra of PPL fractions of Óbidos Lagoon site 3 (OL site 3). The annotations show the characteristics DOM chemical shift (ppm) ranges for: aliphatic, MDLT (material derived from linear terpenoids), CRAM (carboxyl rich alicyclic molecules), aromatic region.

The results are summarized in Table 7.3 and show that the relative percentage of the four functional groups is: MDLT > CRAM > Carbohydrate/protein > Aromatic for these forms of DOM. The %area of the carbohydrate/ protein and MDLT regions showed the greatest variation. The %area of the MDLT region was positively correlated with increasing eluent hydrophobicity while the %area of the carbohydrate/protein resonance decreased with the increase in eluent hydrophobicity. The %MDLT is highest at OL site 1 and lowest at site 3 in all three PPL fractions. OL site 1 and the NPBB have the same % composition of MDLT in their corresponding PPL I and PPL II fractions. However, PPL III from the NPBB has the highest percentage MDLT (63%) of any PPL fraction. The MDLT content of the IRWD wetland water was similar to OL sites 1 and 2. The MDLT content of SRNOM was most similar to that of OL site 3.

Table 7.3 – ^{1}H NMR results (integration areas) for the PPL extracts obtained for Óbidos Lagoon (OL), Newport Back Bay (NPBB), IRWD SJ Wetlands (IRWD) and the standard Suwannee River natural organic matter (SRNOM) ((% Area = Area of each group/sum of areas)*100).

	Aromatic	Carb/prot	CRAM	MDLT
Chemical shift (ppm)	**8.9 - 6.9**	**6.9 - 3.40**	**3.4 - 1.9**	**1.9 – 1.0**
PPL I				
OL Site 1	6.0	27	31	37
OL Site 2	6.0	24	31	39
OL Site 3	10	27	33	29
NPBB (comb)	3.4	29	33	35
IRWD Inlet	5.7	26	35	34
IRWD Outflow	11	26	27	35
SRNOM	7.9	20	33	28
PPL II				
OL Site 1	0.3	15	37	48
OL Site 2	10	18	30	42
OL Site 3	7.0	17	33	43
NPBB (comb)	0.1	14	37	49
IRWD Inlet	5.0	15	34	47
IRWD Outflow	6.0	16	37	41
SRNOM	9.0	14	26	36
PPL III				
OL Site 1	2.2	11	30	57
OL Site 2	10	13	29	48
OL Site 3	7.0	12	32	50
NPBB (comb)	3.2	8.0	26	63
IRWD Inlet	9.0	13	30	48
IRWD Outflow	3	10	38	49
SRNOM	1	9	26	54
SRNOM bulk	**11.1**	**21.0**	**32.0**	**30.4**

The PPL I fraction had the highest %carbohydrate/protein content and was approximately 27% of the total proton resonance for the natural waters and was 20% for SRNOM. In general the percentage of carbohydrates is higher in marine waters (deep and surface ocean) than in freshwaters [44, 58]. The CRAM region was highest in the PPL II fraction for OL sites 1 (37%) and site 3 (33%), and the NPBB (37%). CRAM was approximately 31% of OL sites 1, 2 and 3. The distribution of CRAM in the IRWD inlet water was 35%:34%:30% but 27%:37%:38% in the outflow water. These observations are consistent with proposal that CRAM is derived from biomolecules in marine

and freshwater [60]. The results suggest that for marine samples with low inputs of terrestrial DOM, higher amounts of CRAM would be found in the intermediate hydrophobic fraction PPL II while for samples with high inputs of terrestrial DOM like the inlet of IRWD SJ wetlands and the SRNOM, higher percentage of CRAM would be found in the PPL I extract. However, further work is needed to confirm the observed trend since numerous structurally different components are likely contributing to the signals of the CRAM region that might have different hydrophobic character. There were no clear trends in the elution patterns of the aromatic fraction of these fractions due to the low proton resonance. OL sites 2 and 3 aromatic content (7 – 10%) was similar in all three PPL fractions. The IRWD had a similar percentage of aromatic resonances. NPBB was more similar to OL site 1 and the relative proportions in all waters were similar to SRNOM.

7.3.2.2 FT-IR spectroscopy

FT-IR spectra of the unfractionated and fractionated PPL I, PPL II and PPL III of SRNOM are shown in Fig. 7.4. The spectra of the PPL fractions of OL, NPBB and IRWD are presented in the supplemental information (Annex D, Fig. D.9 and Fig. D.10). The major bands present in the spectra of the PPL fractions and the corresponding vibration type are summarized in Table 7.4.

The spectrum of the unfractionated SRNOM (Fig. 7.4) shows several broad bands due to overlapping signals, which is typical of natural organic matter [61, 62]. The very broad band centered at ≈3300 cm^{-1} can be attributed to the overlap of O-H stretching of phenol, carboxylic acids, carbohydrates and N-H stretching of amides [62]. The band between 2970 – 2830 cm^{-1} is related with the stretching vibrations of C-H bonds in methylene (CH_2) and methyl (CH_3) groups of aliphatic chains of carbohydrates, proteins and lipids [61-63]. A weak band in the range of 2510 – 2610 cm^{-1} is typical of the S-H stretching of a thiol functional group [63, 64]. The intense band at 1718 cm^{-1} is characteristic of the stretching vibration of the carbonyl group (C=O) present in the carboxylic acids, ketones, aldehydes and esters [62, 64, 65]. The band

with a peak at 1618 cm^{-1} can be due to the overlap of the aromatic C=C stretching and C=O stretching of quinone and/or conjugated ketone and amide groups as well as the N-H bending of amides [61, 66, 67]. The shoulder at 1389 cm^{-1} can be due to the simultaneous symmetric stretching of the C=O of carboxylic acids and asymmetric stretching of C-N amides as well as the C-H deformation of aliphatic and CH_3 groups (aliphatic hydrocarbons) and the O-H in plane bending of carbohydrates [61-63, 68]. The band centered at 1043 cm^{-1} with two shoulders at 1198 cm^{-1} and 962 cm^{-1} can be ascribed to the overlap of the C-O asymmetric stretching of aromatic compounds like lignin and also the O-H out-of-plane bend of carboxylic acids and symmetric C-O stretching of alcohol, phenols and carbohydrates/ polysaccharides [61-63, 65]. The 900 – 880 cm^{-1} band is caused by out of plane deformations of the hydrogen atoms present in aromatics and/or alkenes [61]. The band between 840 – 730 cm^{-1} is related with the aromatic C-H bends [61, 69].

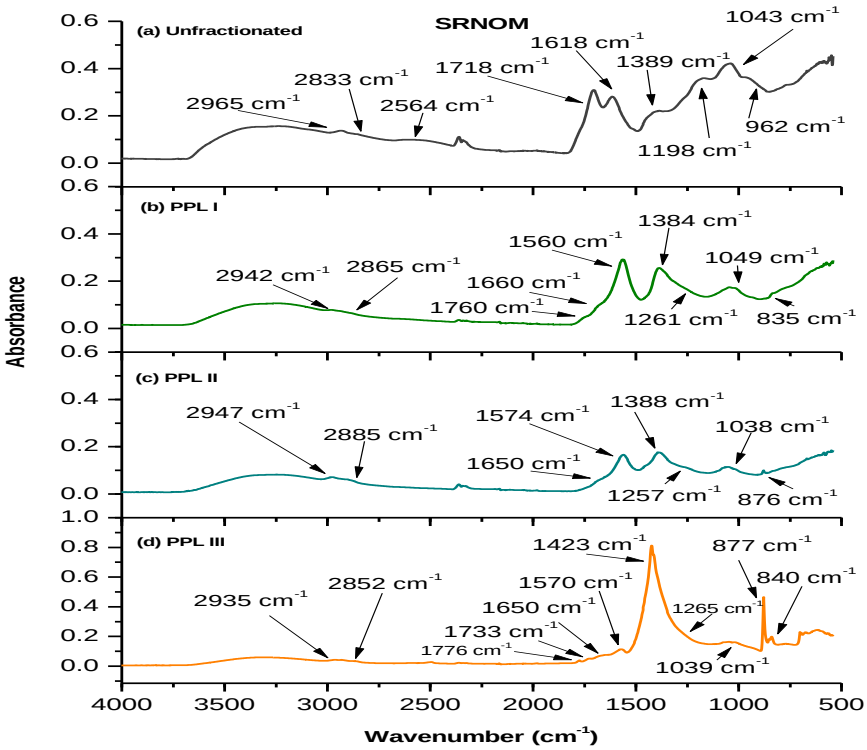

Fig. 7.4 – FT-IR spectra of the (**a**) unfractionated SRNOM and its corresponding PPL fractions: (**b**) PPL I = MeOH:H_2O (1:1), (**c**) PPL II = MeOH:ACN:H_2O (1:1:2); (**d**) PPL III = MeOH:ACN (1:1).

As shown in Fig. 7.4, the spectra of the PPL extracts of SRNOM are more highly resolved than the corresponding unfractionated sample. In general, the intensity of the broad band (centered ≈3300 cm^{-1}) responsible for the O-H stretching of phenol, carboxylic acids and carbohydrates decreased from PPL I to PPL III. The intensity of the band assigned between 1043 – 989 cm^{-1}, which is characteristic of the presence of carbohydrates/polysaccharides also followed the same trend. This result is in accordance with the ^{1}H NMR analysis that showed a decrease of the %carbohydrate with an increase of eluent hydrophobicity. The band assigned between 1043 – 989 cm^{-1} is also more intense for marine samples with a low terrestrial input such as OL site 1 and NPBB. Marine DOM is believed to be the decay products of phytoplankton and consists of 25 – 50% proteins, 5 – 25% of lipids and up to 40% of carbohydrates [44, 68].

The band centered between the 1587 – 1553 cm^{-1} can be caused by an overlap of the vibrations of deprotonated carboxylic groups, secondary amides, esters and aliphatic structures. The band is less intense in the more hydrophobic PPL fraction (PPL III). The shoulders assigned at 1753 – 1733 cm^{-1} and 1660 – 1652 cm^{-1} that are related with the asymmetric stretching of C=O of esters and primary amides, respectively, also showed a decrease in the PPL III extract. The presence of the carboxylates in the PPL fractions instead of the carboxylic group is due to the addition of NaOD during the NMR analyses. NMR is a non-destructive technique that was carried out before FTIR analysis. A pH raise converted most of the -COOH group into its salt form ($-COO^{-}\ Na^{+}$).

The band centered at 1388 - 1383 cm^{-1} is related with the C=O symmetric stretching of deprotonated carboxylic acids. The overlap of the vibration of amides, aromatic and aliphatic structures are indicated by the presence of the shoulders assigned at 1459 – 1456 cm^{-1} and 1250 – 1240 cm^{-1} (Table 7.4). The band centered at 1388 - 1383 cm^{-1} became broader with an

increase of terrestrial inputs from OL site 1 to OL site 3 and was less intense in PPL III of site 1. For PPL III fraction of OL site 2, NPBB and SRNOM the 1388 - 1383 cm^{-1} band was substituted or overlapped by an intense band assigned between 1425 – 1421 cm^{-1}. This band can be attributed to the C-H bend of unsaturated aliphatic compounds along with the stretching of C-N bond of amides (Table 4). Amides and unsaturated aliphatic compounds are likely to be present due to the simultaneous occurrence of the bands centered between 1664 – 1650 cm^{-1} and 881 – 879 cm^{-1}, respectively (Table 7.4). The results of the EEMs integration for the PPL III fraction of those samples showed an increase of the %total fluorescence for the protein-like T peak and a decrease of the other peaks (Table 7.2). The increase in the %total fluorescence of the protein-like T peak was more pronounced in the PPL III fraction of NPBB. This fraction also showed a high content of MDTL (63%, Table 7.3). Both facts seem to be reflected in the shape and the intensity of the 1425 cm^{-1} band.

The band centered between the 1425 – 1421 cm^{-1} is present as a major band in all PPL fractions of IRWD inlet and outflow (Fig. D.10). The results of the ^{1}H NMR showed an increased of the %MDTL and a decreased of %CRAM along the PPL fractions of the IRWD inlet (Table 7.3). The PPL III fraction of the inlet also showed an increased of the %total fluorescence of the protein-like T peak, which can justify an increase of the 1425 cm^{-1} band intensity (Fig. D.10). The PPL fractions of the IRWD outflow showed an increased of the %MDTL and %CRAM. The outflow PPL III fraction also showed an increase of the %total fluorescence of the protein-like T peak, however, the humic-like A peak also increased which might contributed to the broadening of the 1425 cm^{-1} band.

The small band assigned between 1776 – 1770 cm^{-1} was present together with both bands 1425 – 1421 cm^{-1} and 881 – 877 cm^{-1}. The band at 1776 cm^{-1} was assigned to the C=O stretching of γ-lactone a five membered ring ester [62]. Biodegradation of terpenoids is a potential source of γ-lactone [62]. Terpene-like compounds or terpenoids are formed by plants,

phytoplankton and bacteria [70]. Terpenoids are hydrocarbon-based natural products whose structure is derived from isoprene [70, 71]. The results suggest that the PPL fractions of IRWD wetlands and the PPL III fraction of OL site 2, NPBB and SRNOM are potentially richer in more conjugated terrestrial aliphatic material due to natural source input from higher plants biomass. The bands 1776 – 1770 cm^{-1}, 1425 – 1421 cm^{-1} and 881 – 877 cm^{-1} are therefore potential markers of MDTL terrestrial inputs. The band 835 cm^{-1} that appears in most of the FT-IR spectra has been assigned to lignin-like compounds together with other bands like 1515, 1450, 1371 and 1265 cm^{-1} [67, 69, 72].

Table 7.4 – Frequency (cm^{-1}) of the major bands identified in the PPL fractions (PPL I, PPL II and PPL III) of Óbidos Lagoon (OL site 1, 2 and 3), Newport Back Bay (NPBB), SJ IRWD (inlet and outflow) and SRNOM. The band position includes in a range the assigned peaks of all the PPL fractions characteristic of each type of vibration.

Band Position (cm^{-1})	Diagnostic Band* (cm^{-1})	Type of Vibration	Assignment
3300	3600 - 3000	O-H and N-H stretching	Carboxylic acids, alcohol, phenol, amines
2976 - 2848	2960 - 2850	C-H stretching: symmetric and asymmetric	Saturated aliphatic chains
1776 - 1770	1775 - 1770	C=O asymmetric stretching	Five member ring ester (γ-lactones)
1756 - 1733	1750 - 1725	C=O asymmetric stretching	Aliphatic esters
1660 - 1652	1680 - 1630	C=O asymmetric stretching	Amides
	1680 - 1620	C=C alkenyl stretching	Unsaturated aliphatic compounds
1587 - 1558	1650 - 1550	N-H bending	Secondary amine
1587 - 1558	1610 - 1550	C=O asymmetric stretching	Carboxylate (carboxylic acid salts)
1459 - 1456	1470 - 1450	Methyl (CH_3) C-H asymmetric bend	Saturated aliphatic compounds
1425 - 1421	1430 - 1390	C-N stretching	Amides
	1420 - 1410	Vinyl C-H bend (symmetric)	Unsaturated aliphatic compounds
1388 – 1383	1420 - 1300	C=O symmetric stretching	Carboxylate (carboxylic acid salts)
	1380 - 1370	Methyl (CH_3) C-H symmetric bend	Saturated aliphatic chains
1307 - 1274	1310 - 1290	Vinylidene C-H in plane bend	Unsaturated aliphatic chains
1250 - 1241	1300 - 1240	C=O stretching	Amides
1125 - 1012	1100 - 900	C-O symmetric stretching	Alcohol, phenol, ethers, carbohydrates
881 - 877	895 - 885	Vinylidene C-H out of plane bend	Unsaturated aliphatic compounds
840 - 831	840 - 730	C-H bending	Aromatic compounds, lignin-like compounds

* References: [61-62, 66-68]

7.3.3 RP-HPLC

The three PPL fractions each generated a characteristic profile that was similar for all water sources (Figs. 7.5 – 7.7) and to the SRNOM (Fig. 7.8). The PPL I chromatogram with gradient 1 (Table D.1) (Figs. 7.5a – 7.8a) was characterized by a broad peak with RT = 8 – 28 min and a single sharp peak (RT = 30 min) that eluted as the gradient was increased to 25% acetonitrile. The broad peak was highest in the SRNOM PPL I making it a characteristic of freshwater DOM. Gradient 2 (Table D.2) was used for the separation of two more hydrophobic fractions (PPL II and PPL III). PPL II generated one major peak (RT = 15.5 min) that eluted with 25% acetonitrile and two minor peaks (RT = 8.0 and 22.5 min). PPL III also generated one major peak (RT = 24 min) that eluted with 50% acetonitrile and minor peaks with retention times less than 20 min. For OL PPL III fraction a sharp peak was obtained at 22 min. While the overall profiles were similar among the different source waters, the more hydrophobic PPL fractions exhibited the largest differences.

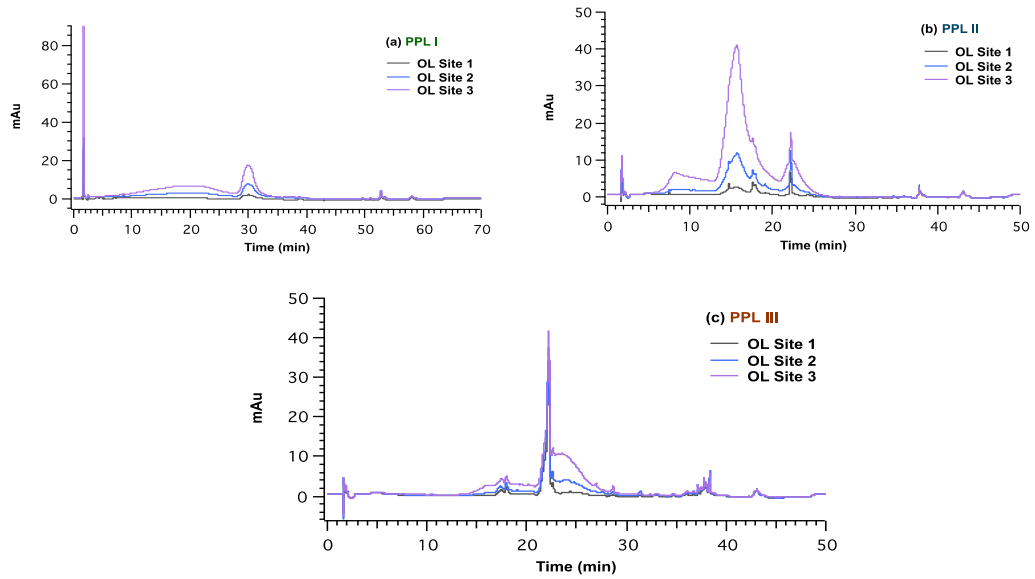

Fig. 7.5 – RP-HPLC chromatograms of the PPL extracts of Site 1, 2 and site 3 of Óbidos Lagoon (OL) obtained with a DAD at 254 nm (mAU): (**a**) Gradient 1 for PPL I: MeOH: H_2O (1:1); (**b**) Gradient 2 for PPL II: MeOH:ACN: H_2O (1:1:2); (**c**) Gradient 2 for PPL III: MeOH:ACN (1:1).

The peak heights of the PPL extracts of OL increased from site 1 (ocean inlet) to site 3 (upper lagoon). This same increase was observed in the UV-Visible absorbance (Table 7.1) and total fluorescence (Table 7.2) and is most probably a consequence of the higher terrestrial inputs through soil runoff, freshwaters tributaries and/or sediments re-suspension that occur in Óbidos Lagoon, especially at site 2 and site 3 [50].

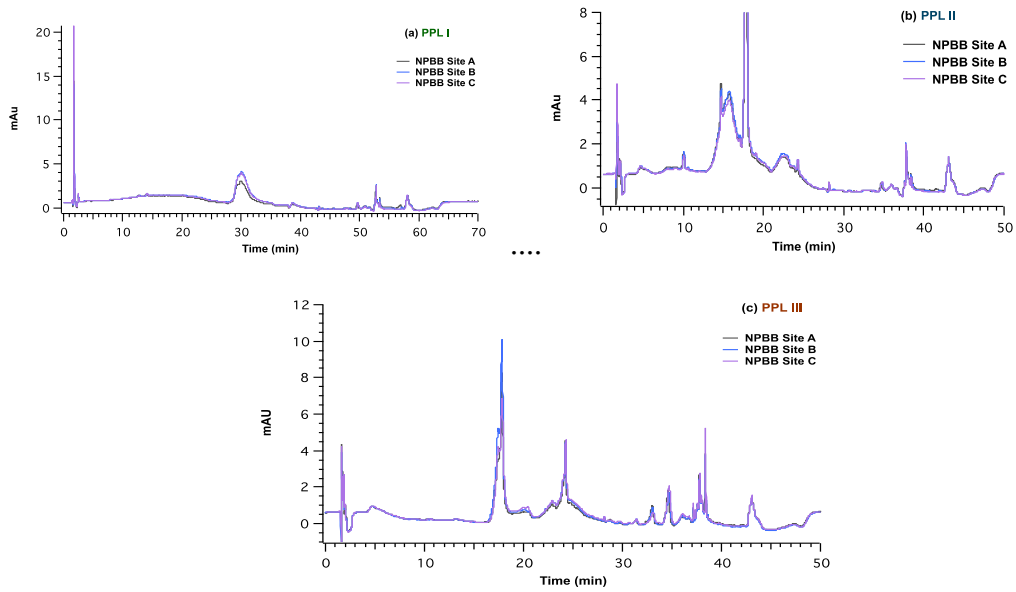

Fig. 7.6 – RP-HPLC chromatograms of the PPL fractions of site A, B and site C of Newport Back Bay (NPBB) obtained with a DAD at 254 nm (mAU): (**a**) Gradient 1 for PPL I: MeOH: H_2O (1:1); (**b**) Gradient 2 for PPL II: MeOH:ACN: H_2O (1:1:2); (**c**) Gradient 2 for PPL III: MeOH:ACN (1:1).

The Newport Back Bay (NPBB) chromatograms for the PPL I, II, and III extracts are shown in Fig. 7.6. Unlike the OL sites; there was no significant difference in UV absorbance (Table 7.1), total fluorescence (Table 7.2) and peak height among the three sites (Fig. 7.6a,b,c). All three PPL extracts have similar profiles to those from OL site 1. The chromatogram of the NPBB PPL I extract (Fig. 7.6a) was almost identical to that from OL site1 (Fig. 7.5a). The PPL II extract of the three NPBB sites were also similar in that they had one main peak (RT = 15.5 min) but had an additional sharp peak (RT = 17.7 min) was superimposed on the main peak. The profile of the PPL III fraction is also similar to that of OL site 1 (Fig. 7.5c) but with an additional peak with RT =

24.4 min. The peak with the reduced height (RT = 17.5 min) is mostly likely carryover from NPBB PPL II. The very sharp peak superimposed on the main peak (RT = 24 min) was not observed in the extracts from any of the OL sites. The presence of these two peaks in only the NPBB water suggests that they may have an autochthonous source or an anthropogenic origin. The same can also be applied to the sharp peak found for PPL III of OL. The peaks with RT> 35 min peaks were not taking into account because they were also detected in the blank samples.

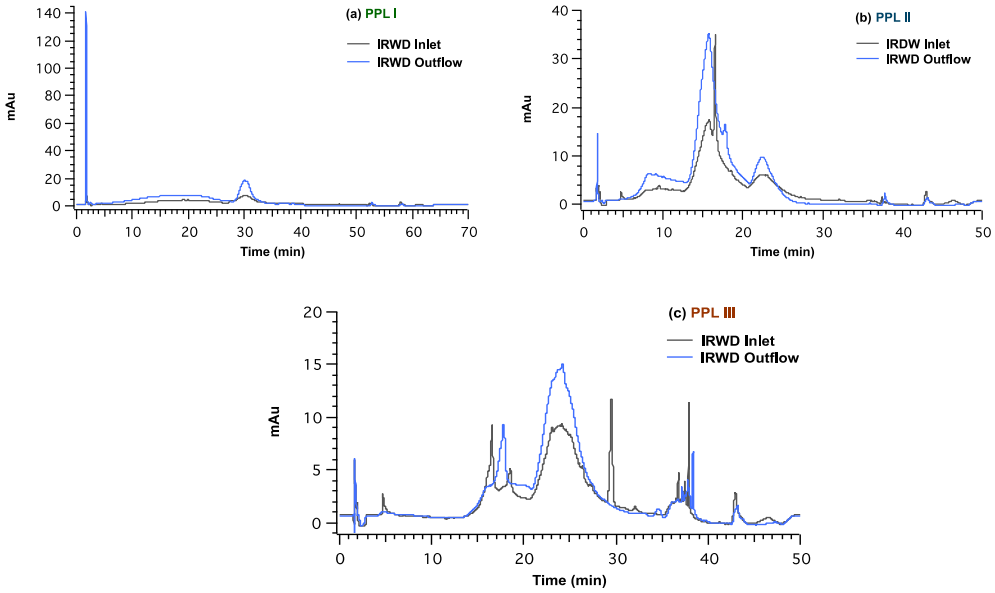

Fig. 7.7 – RP-HPLC chromatograms of the PPL extracts of inlet and outflow of IRWD SJ wetlands obtained with a DAD at 254 nm (mAU): (**a**) Gradient 1 for PPL I: MeOH: H_2O (1:1); (**b**) Gradient 2 for PPL II: MeOH:ACN: H_2O (1:1:2); (**c**) Gradient 2 for PPL III: MeOH:ACN (1:1).

The chromatograms of the PPL extracts of the inlet and outflow from the IRWD wetlands are shown in Fig. 7.7. The UV absorbance (Table 7.1), the total fluorescence and the chromatogram peak heights (Fig. 7.7) of the inlet are less than that of the outflow. The profiles of the PPL I extracts (Fig. 7.7a) are similar to those of both the Óbidos Lagoon and Newport Back Bay. The profiles of the PPL II extracts (Fig. 7.7b) are also very similar but the IRWD inlet extract has an additional well-resolved peak (RT = 16.9 min) superimposed on the main peak. The chromatograms of the PPL II and PPL

III extract of IRWD inlet and outflow (Fig. 7.7b, Fig. 7.7c) are similar to that of SRNOM (Fig. 7.8b, Fig. 7.8c).

In general, PPL I and PPL III fractions shows the greatest compositional variation depending the water source. PPL I of riverine waters and the SRNOM standard with terrestrial input show a broad peak that elutes from 5 - 20 min. This peak is significantly reduced in the marine waters with low terrestrial input. The same trend was observed in the PPL III fraction at RT = 24 min. This peak increases from marine to freshwaters and SRNOM. PPL III is richer in the more hydrophobic compounds because the peaks elute later in the gradient. There is some peak overlap between the PPL extracts that could be improved either by re-extraction or by using with a larger volume of eluent. Preparative HPLC and fraction collection would be needed in order to do analyse the EEMs fluorescence and UV-visible spectra the HPLC fractions.

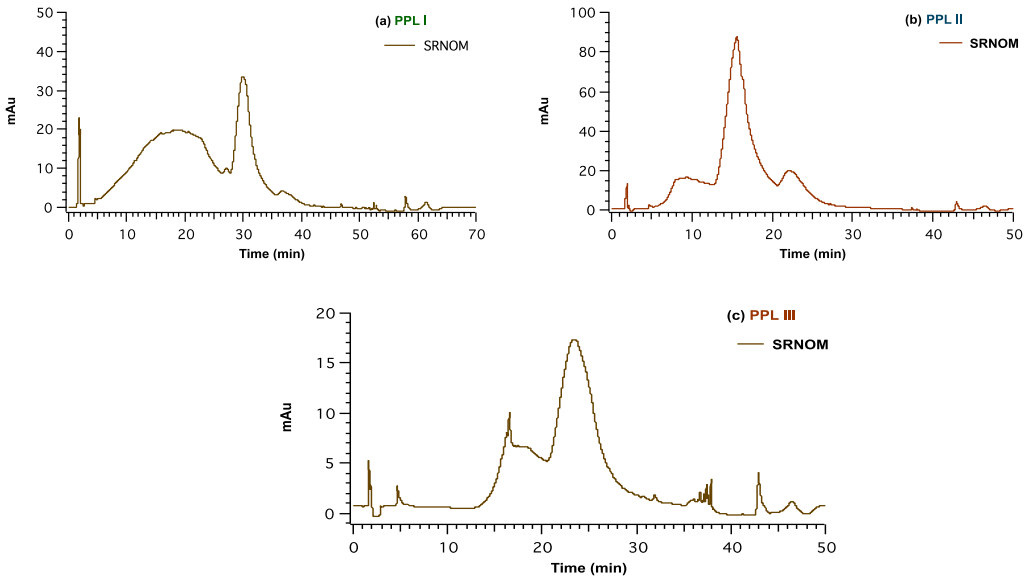

Fig. 7.8 – RP-HPLC chromatograms of the PPL extracts of SRNOM obtained with a DAD at 254 nm (mAU): (**a**) Gradient 1 for PPL I: MeOH:H_2O (1:1); (**b**) Gradient 2 for PPL II: MeOH:ACN: H_2O (1:1:2); (**c**) Gradient 2 for PPL III: MeOH:ACN (1:1).

In summary the RP-HPLC chromatograms show that: (a) the use of a stepwise gradient for the PPL extraction of DOM results in a selective fractionation of the DOM based on the polarity; (b) the broad peak observed in the RP-HPLC chromatograms of the PPL I and PPL III extracts differentiates between fresh and marine waters; (c) while the overall profile of the PPL II extracts are very similar, the highly resolved peaks may be markers of either anthropogenic contaminants or of terrestrial runoff; (d) this fraction scheme is amenable to LC-MS and on-line NMR and FT-IR analysis [73].

7.3.4 Major characterization of DOM from coastal and wetlands

In this study an elution SPE gradient approach was used to fractionate DOM from a lagoon, an estuary and wetland waters. The Óbidos Lagoon (OL), located on the Western coast of Portugal, is a well-characterized meso-tidal lagoon with two upper regions influenced by terrestrial runoff and a tidal influenced near-coastal region [50]. Terrestrial input increases from inlet towards the inner parts of the lagoon. This terrestrial input was reflected on the UV-Vis spectroscopic responses as well as on the RP-HPLC chromatograms with as increase of the respective absorbance from OL site 1 to OL site 3. The PPL extracts of OL show differences in fluorescence based on location and hydrophobicity. Peak T was significantly higher in the PPL III fraction at OL site 1 and OL site 2 suggesting a different source for the fluorescence. The PPL III of OL site 2 also show differences in the FT-IR spectrum with an increase of highly conjugated aliphatic compounds probably as a result of terpenoids degradation. These results suggest the input of a different type of terrestrial DOM (more conjugated aliphatic compounds) at OL site 2 that does not happen at OL site 3. These results also show a spatial variation of DOM composition inside the lagoon which might have consequences on water quality and ecology [74].

The Newport Back Bay (Newport Beach, CA, USA) is an urbanized tidal saltwater wetland comprised of an upper region that is an ecological

preserve and coastal outlet influenced by local marinas [75]. It is the discharge point for the Newport Bay Watershed and is the largest estuary in Southern California. The San Joaquin Marsh and Wildlife Sanctuary (Irvine Ranch Water District - IRWD, Irvine, CA USA) wetlands remediate inorganic nutrients in the urban runoff before entering the Newport Back Bay. The results of DOM PPL fractions from Newport Back Bay (NPBB) were in general identical to OL site 1. The influence of DOM from the IRWD wetlands was showed through PPL II and PPL III extract. The occurrence of a FT-IR band characteristic of highly conjugated aliphatic compounds that was also present in the PPL extracts of IRWD suggests an input of DOM from the wetlands. The PPL fractions of the IRWD outflow wetland showed higher responses than the inflow. The PPL III fraction showed extra peaks in the RP-HPLC chromatogram and the bands of the FT-IR spectrum were less broad. The results showed an increase in lower molecular weight structures through the wetland, perhaps due to DOM photodegradation. The SRNOM PPL fractions showed a similar chromatographic behaviour of the IRWD wetland. Differences occurred in the FT-IR results of the PPL III fraction.

The analysis of the PPL fractions allowed a better characterization of DOM present in the different types of waters especially in terms of sources. PPL differences were found in the fluorescence, NMR and FT-IR. The coupling of the developed herein fractionation and RP-HPLC separation method to those or other type of analytical techniques will narrow the complexity allowing for an in-depth chemical characterization of DOM PPL fractions.

7.4. Conclusions

The developed SPE-based methodology enabled a selective fractionation of dissolved organic matter (DOM) based on eluent hydrophobicity. The application of a range of spectroscopic analytical techniques demonstrated that the sequentially eluted DOM extracts were

different in terms of polarity, chemical composition and inherent molecular weight. The more hydrophobic fractions were enriched with material derived from linear terpenoids (MDTL) while the less hydrophobic fractions showed a high percentage of carbohydrates. The aromatic and the carboxylic-rich alicyclic molecules (CRAM) showed a high variability among the different fractions depending on the type of water. The developed methodology also showed to be useful in the identification of possible sources of DOM. The FT-IR spectra of the DOM fractions obtained from the wetlands revealed that the vibrational bands 1776, 1425 and 881 cm^{-1} are potential markers of the presence of allocthonous highly conjugated aliphatic structures in in marine and freshwaters. The improved RP-HPLC methodology confirmed that the DOM fractions are different in terms of polarity and allowed the identification of specific peaks/zones in the chromatograms that might be used as a proxy of terrestrial DOM input. The developed fraction scheme showed to be simple and selective and is amenable to on-line NMR and FT-IR analysis allowing for an in-depth characterization of the more highly resolved DOM.

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8

Final Considerations

8.1 General discussion

Pesticides are a vast class of compounds and their behaviour in the environment is essential for a better understanding of their fate and impact on aquatic organisms and human health. The results of the scientific studies included in this thesis aim to answer the following five relevant questions concerning pesticide concentration, sources, pathways and impact on aquatic organisms especially in coastal lagoons:

1. Within the vast class of pesticides and the current constrains on the analytical capacity, which should be considered a prioritization in monitoring programs including water, sediment and biota?
2. Does the performance of the standard analytical methods comply with the established limits of concentration of priority pesticides (PPs) in waters, sediments and biota?
3. What are the main sources of PPs in coastal lagoons and how pesticides tend to be spatially distributed in sediments (the example of Óbidos Lagoon)?
4. When extensive macroalgae biomass covers the sediment of the lagoons, do they accumulate the pesticides entering the lagoon?
5. What are the effects of dissolved organic matter (DOM), namely from terrestrial inputs, in the fate of pesticides?

In order to address the first two questions a review concerning the control of pesticides and the performance criteria for their measurements in waters was undertaken (**Chapter 2**). The review shows that under the Water Framework Directive (WFD) a particular group of pesticides that are considered toxic, persistent and liable to bio-accumulate are prioritized in water, sediments and biota monitoring programs [1, 2]. Such group of pesticides herein defined as priority pesticides, were therefore selected as the target compounds due to their toxicity and relevance for the evaluation of the good chemical status of the water bodies at an European level. The review presented in **Chapter 2** shows that the existing standard analytical methods

used for the determination of PPs in water samples are not sensitive enough to comply with the established environmental quality standards (EQSs) and the technical specifications setup for their measurements in water samples. Laboratories must rely on their own analytical methods implying extra efforts for their development and maintenance of the associated quality control and quality assurance. The review also points out the recent innovations in the liquid-phase microextraction (LPME) based techniques. Although not applied in any of the experimental part of the presented studies the review of the LPME based techniques had its importance in the evaluation of the state of art of PPs monitoring in waters. Most of the PPs are extremely hydrophobic and their presence in surface waters is at trace levels in the range of the ngL^{-1}. In the last years efforts have been made to enhance sensitivity of the analytical methods used for water analysis. Although WFD recommends that sediments and biota shall be the preferable matrix for the analysis of highly hydrophobic priority substances (log k_{ow}>3) less attention has been paid to the determination of such compounds in sediments in comparison to water [1, 2]. A reliable estimation of the overall amount of contaminated sediments in Europe is still hard to give mainly due to absence of uniformity in the sampling methods, analytical techniques and applied sediment quality standards or guidelines values [3]. No standard methods are available for the determination of multi-residue/multiclass pesticides in sediments. This thesis presents an analytical methodology that was applied for the determination of the entire list of the most hydrophobic PPs (**Chapter 3**). In the absence of performance criteria for the determination of PPs in sediments the analytical outputs were compared to the guidelines used for food and feed analysis [4]. Besides its good precision, accuracy and lower limits of quantification, the method showed to be robust since it can be applied to different types of sediments like sand and muddy.

As referred in **Chapter 1**, Barrosa branch is an area that has been classified as a sensible zone relatively to eutrophication problems. The

massive growth of seaweeds in that area raised the hypothesis that *Ulva* sp. pesticides would accumulate the PPs. To test this hypothesis (4th question) an analytical methodology was firstly developed for the determination of PPs in *Ulva* sp (**Chapter 4**). Improved performance for a high number of pesticides was achieved by application of a selective pressurized liquid extraction (SPLE) technique. The founded results suggest that *Ulva* sp. seaweeds tend to accumulate some PPs especially after a long period of rainfall. This type of macroalgae is thus a promising analytical matrix for the evaluation of pesticide contamination by runoff working as early alert signal of aquatic pollution. The accumulation of the PPs by the *Ulva* sp. after extensive periods of storms illustrate an important issue about the effects of the climate change in the dissipation of the pesticides in coastal lagoons. Depending on the emissions scenarios, predictions point out for a global temperature change by 2100, compared to 1990, between 0.3 to 6.4 ºC [5]. The global warming phenomenon is also predicted to change the hydrological cycle and increase precipitation in northern and central Europe [5]. Extreme events, such as storms are also predicted. These will increase the flooding risk in the lagoon systems [5, 6]. All this changes are likely to increase pesticide concentration in the aquatic systems due to stronger soil runoff caused by an increase in precipitation and flooding. Studies indicate that climate change, especially sea level rise and global warning, are likely to affect shallow coastal lagoons and to increase their vulnerability to eutrophication [5, 6]. Global warming will probably have an effect on pesticides solubility raising their concentration on water column and thus their transport and also their bioavailability towards aquatic organisms like *Ulva* sp.. Results of **Chapter 4** show the importance of the studies carried out in lagoons and how the uptake of pesticides by aquatic organisms can be influenced by adverse climate conditions.

The outcomes of **Chapter 5** answered the question raised about the sources and pathways of pesticides in Óbidos Lagoon. Results of **Chapter 5** provided a more realistic image of the PPs accumulation and distribution

along Óbidos Lagoon. Agricultural uses as well as livestock were found to be the major source of the PPs found in Óbidos Lagoon in accordance with the main pressures identified for that area. Soil run-off is the principal pathway of entrance in the lagoon, although for some PPs their occurrence can be due to discharge of potentially contaminated small tributaries that enter into the lagoon. The PPs present in Óbidos Lagoon are in the range of 2.2 to 35 ngg^{-1} (dry weight). They were confined to *Barrosa* branch as a consequence of the longer residence time of the water and its weak currents that favors particle deposition. The results presented in **Chapter 5** also show that sediment monitoring is essential for the control of recent inputs of PPs. Among the studied pesticides, lindane, *p,p'*-DDT and the metabolite heptachlor epoxide were found to be at concentrations above the ''probable effect level''. Adverse effects of those PPs on aquatic organisms are thus expected to occur frequently [7].

To address the fifth question studies were carried out with chlorpyrifos (**Chapter 6**). This pesticide has a higher water solubility when compared to the other PPs and is still worldwide applied and therefore it is of importance. Through **Chapter 6** this thesis introduced for the first time the impact of DOM on the dissipation and transformation of chlorpyrifos in the saline environments. In coastal lagoons both volatilization and photolysis show to be important pathways of chlorpyrifos loss. The addition of terrestrial DOM to mimic the mixing of freshwaters with seawaters increased the photodegradation rate constant of chlorpyrifos in waters with high salinity. Volatilization had an opposite trend decreasing with the addition of DOM probably due an increase of its apparent solubility. These results show the importance of DOM in the partition of the PPs in coastal lagoons. The results of this **Chapter 6** also suggest that water turbulence and DOM can be important aspects to take into account in the evaluation of the impacts of climate change on coastal lagoons and pesticide fates. Volatilization of chlorpyrifos increased with an increase of the aeration of the system. Storms

events will increase water turbulence and are likely to influence partition of chlorpyrifos and other PPs between water phase and the atmosphere. A decrease of salinity and an increase of temperature will also favor pesticide solubilities. High inputs of natural organic matter (NOM) as a consequence of stronger soil runoff (due to storms and precipitation) will probably increase pesticides solubility and their concentration in the aqueous phase. An increase of NOM can also influence the photodegradation rate of the pesticides. The adverse impact of this process on the aquatic organisms will depend on the toxicity and the concentration of their degradation products.

The effectiveness of DOM in enhancing compounds solubility appears to be largely controlled by DOM source, its molecular size and also polarity [8, 9]. DOM is a complex mixture of thousands of compounds and its fractionation according to its properties would narrow its complexity. In this sense, an analytical study was conducted to fractionate DOM from different types of waters including coastal lagoons. The study presented in **Chapter 7** introduced a simple and innovative approach for the fractionation of DOM. The new SPE-RP-HPLC methodology enabled a selective fractionation of DOM based on eluent polarity. Significant differences were obtained in the chromatographic profiles among the main DOM fractions. The method showed to be robust and has the potential to be used as a proxy to differentiation between fresh and marine waters. The developed methodology can be coupled to a wide range of detection techniques allowing in the future for an in-depth characterization of the more highly resolved DOM.

Overall, this thesis points out new and important data in the field of pesticides fate in coastal lagoons. Analytical methods for PPs sediment analysis by GC-MS were developed that fulfill a gap in this area. The main sources and pathways of the PPs in Óbidos Lagoon were identified as well as the zones of environmental concern relatively to pesticide contamination that might have an impact on the aquatic organisms in the future.

8.2 Future work

The work performed in this thesis provided the basis for future and relevant research issues. These issues include:

i) The screening of the PPs in biota especially in the areas identified as of environmental concern like the *Barrosa* branch.

ii) A long-term screening of the PPs and other relevant pesticides in sediments, *Ulva* sp. seaweeds and biota after extensive periods of heavy rains to evaluate their accumulation and uptake under adverse climactic conditions. The list of pesticides shall be extended to the other PPs that have been added in the latest update of the list of priority substances (Annex A, Table A.1).

iii) The evaluation of the effect of the DOM fractions isolated by SPE gradient elution on pathways of the PPs, particularly chlorpyrifos.

iv) The development of certified sediment reference materials for the entire list of the priority pesticides that will enable a better evaluation of the accuracy of any analytical method.

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Annexes

Supplementary Information

Supplementary Information - Annex A

Chapter 1

Table A.1 – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33] (references of Chapter 1).

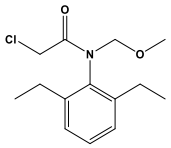
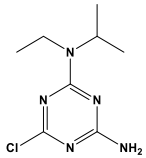
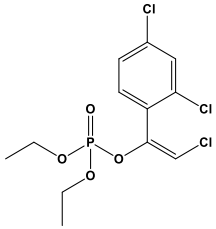
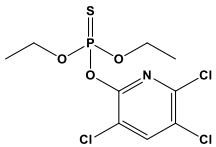
Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log Kow	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification
Alachlor $C_{14}H_{20}ClNO_2$ CAS** [15972-60-8] Chloroacetamide Herbicide		269.8	1200	3.52	1.87-4.13 (20-25 °C)	1.21×10^{-5} - 3.2×10^{-3} (20-25 °C)	In water 148-242 mgL^{-1} (25 °C). Soluble in diethyl ether, acetone, benzene, chloroform, ethanol and ethyl acetate. pK_a 1.60.	Selective systemic herbicide. Use pre-emergence to control annual grasses and many broad-leaved weeds in cotton, maize, peanuts, beans, sugar cane, etc.	PP listed in WFD as group (1)
Atrazine $C_8H_{14}ClN_5$ CAS [1912-24-9] 1,3,5-triazine Herbicide		215.7	1869	2.75 (25 °C)	0.040 (25 °C)	2.88×10^{-4} - 1.00×10^{-3} (20-25 °C)	In water 28-70 mgL^{-1} (25 °C). In ethyl acetate 24, acetone 31, dichloromethane 28, ethanol 15, toluene 4.0, *n*-hexane 0.11, *n*-octanol 8.7 (all in gL^{-1}).	Selective systemic herbicide. Pre and post-emergence control of annual broad-leaved weeds and annual grasses in maize, sorghum, sugar cane, pineapples, and golf courses.	PP listed in WFD as group (3)
Chlorfenvinphos $C_{12}H_{14}Cl_3O_4P$ CAS [470-90-6] Organophosphorus Insecticide, Acaricide		359.6	12	3.85 (Z) isomer 4.22 (E) isomer	0.027-1 (20-25 °C)	2.8×10^{-4} -0.324 (20-25 °C)	In water 124-145 mgL^{-1} (20-25 °C). Miscible with most common organic solvents.	Insecticide and acaricide with contact and stomach action and with long residual activity. Soil application for control of root flies, rootworms and other soil insects in vegetables; Foliar application for control of Colorado beetles on potatoes; scale insects and mite eggs on citrus fruit and whitefly on cotton. Used in public health for control of mosquito larvae. Also used as an animal ectoparasiticide.	PP listed in WFD as group 8
Chlorpyrifos $C_9H_{11}Cl_3NO_3PS$ CAS [291-88-2] Organophosphorus Insecticide		350.6	135	4.46, 5.27	0.52-4 (20-25 °C)	0.3-1.8 (20-25 °C)	In water 0.30-1.4 mgL^{-1} (20-25 °C) [1]. In benzene 7900, acetone 6500, chloroform 6300, diethyl ether 5100, methanol 450 (all in gKg^{-1}, 25 °C).	Non-systemic insecticide, cholines-terase inhibitor. Used for a wide range of crops, including pome fruit, vines, vegetables, etc. Also used in household pests control and in animal houses. Non-phytotoxic to most plant species when used as recommended.	PP listed in WFD as group 9

Table A.1 (cont.) – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33].

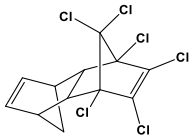
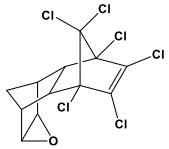
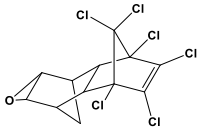
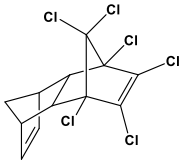
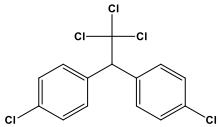
Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log K_{OW}	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification*
Aldrin $C_{12}H_8Cl_6$ CAS [309-00-2] Ciclodiene organochloride Insecticide		364.9	39	5.17-7.4 (25 ºC)	0.9-3.1 (20-25 ºC)	4.46-91.23 (20-25 ºC)	In water 27 μgL^{-1} (20-25 ºC). Moderately to very soluble in most aromatic hydro-carbons, esters, ketones, and halogenated solvents.	Act as highly effective contact and stomach poisons for insects. Used as for the protection of corn, potato, citrus, and other crops against termites, corn rootworms, seed corn beetles and maggots, wireworms, rice water weevil, grasshoppers, etc.	Not a PP listed under WFD as group 9a* POP
Endrin $C_{12}H_8Cl_6O$ CAS [72-20-8] Ciclodiene organochloride Insecticide		380.9	3	5.20	0.027 (20-25 ºC)	0.05-0.76 (25 ºC)	In water 230 μgL^{-1} (20-25 ºC), acetone 170 gL^{-1}; *n*-hexane 710 gL^{-1}	Act as highly effective contact and stomach poisons for insects. It was used to control cutworm, voles, grasshoppers, borers in food and nonfood commodities. It was used to control nuisance birds.	Not a PP listed under WFD as group 9a* POP
Dieldrin $C_{12}H_8Cl_6O$ CAS [60-51-1] Ciclodiene organochloride Insecticide		380.9	40	3.69-6.2 (25 ºC)	0.02-2.4 (20-25 ºC)	0.02-5.88 (25 ºC)	In water 200 μgL^{-1} (20-25 ºC). Moderately soluble in common organic solvents.	Act as highly effective contact and stomach poisons for insects. It was then used principally to protect wooden structures against ant and termite attack and as a residual spray and larvacide for the control of several insect vectors of disease.	Not a PP listed under WFD as group 9a* POP
Isodrin $C_{12}H_8Cl_6$ CAS [465-73-6] Ciclodiene organochloride Insecticide		364.9	24	6.82	10.35	--	In water 17 μgL^{-1} (20-25 ºC)	Act as highly effective contact and stomach poisons for insects. Its insecticidal action is similar to aldrin but more efficient. Although not regulated as a persistent organic pollutant (POP) (like dieldrin and endrin) isodrin has similar properties of toxicity, persistence and tendency to bioaccumulate that characterize POPs.	Not a PP listed under WFD as group 9a*
***p,p'*-DDT** **(dichlorodiphenyltrichloroethane)** $C_{14}H_9Cl_5$ CAS [50-29-3] Organochloride Insecticide		354.5	87	6.36, 6.91	0.025-0.8 (20-25 ºC)	0.86-8.2 (20-25 ºC)	Practically insoluble in water. Readily soluble in aromatic and chlorinated solvents.	Non-systemic insecticide with contact and stomach action. Used as a mosquito vector control for the eradication of malaria. Usage on crops generally been displaced by less persistent insecticides.	Not a PP listed under WFD as group 9b* POP

Table A.1 (cont.) – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33].

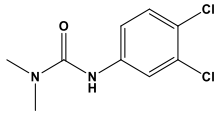
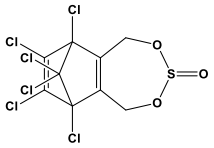
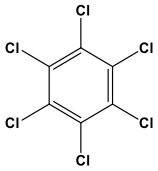
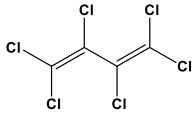
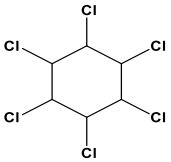
Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log Kow	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification*
Diuron $C_9H_{10}Cl_2N_2O$ CAS [330-54-1] Urea Herbicide		233.1	3400	2.78 (25°C)	1.1×10^{-3}-0.53 (20-25 °C)	1.5×10^{-4} (20-25 °C)	In water 36.4 mgL^{-1} (25°C). In acetone 53 and benzene 1.2 gkg^{-1} at 27 °C.	Systemic herbicide absorbed by roots. Inhibits photosynthesis. Total control of weeds and mosses on non-crop areas. Selective control of germinating grass and broad leaved weeds in many crops (fruits, vine, etc.).	PP listed under WFD as group 13
Endosulfan $C_9H_6Cl_6O_3S$ CAS [959-98-8] α-endosulfan CAS [891-86-1] β-endosulfan Ciclodiene organochloride Insecticide, Acaricide		406.9	18	4.74 α-isomer (pH 5) 4.79 β-isomer (pH 5)	0.083 (20 °C, mixture of α- and β-isomers 2:1)	1.48 (α-isomer) 0.07 (β-isomer) (22 °C)	In water 0.32 mgL^{-1} (20-25°C). In ethylacetate, dichloro-methane, toluene 200, ethanol 65, *n*-hexane 24 (all in gL^{-1}, 20 °C).	Non-systemic insecticide with contact and stomach action. Control of sucking, chewing and boring insects and mites on a very wide range of crops, including fruits, vines, olives, vegetables, potatoes, maize, coffee, rice, cotton, etc. Also controls tsetse flies.	PHS listed under WFD as group 14 POP
Hexachlorobenzene C_6Cl_6 CAS [118-74-1] Organochloride Fungicide		284.8	10000	5.44, 5.73	0.28-1.47 (20-25 °C)	7.2-139 (20-25 °C)	Practically insoluble in water. Soluble in hot benzene, chloroform, carbon disulfide and diethyl ether. Sparingly soluble in carbon tetrachloride.	Selective fungicide. Acts by fumigant action on fungal spores. Used in seed treatment for control of common bunt and dwarf bunt of wheat.	PHS listed under WFD as group 16
Hexachlorobutadiene C_4Cl_6 CAS [87-68-3] Organochloride Insecticide		260.8	200-580	4.78	20×10^3 (20 °C)	1044-2604 (25 °C)	In water 2.6 mgL^{-1}; soluble in ethanol and diethyl ether [WHO]	HCBD was used as a solvent in chlorine gas production, an intermediate in the manufacture of rubber compounds, a lubricant, a gyroscopic fluid and a fumigant in vineyards.	PHS listed under WFD as group 17 POP
Hexachlorocyclohexane (HCH) $C_6H_6Cl_6$ CAS [608-73-1] Organochloride Insecticide		290.8	76	3.80 α and β isomer 4.14 δ-HCH 3.5 γ-HCH	α-HCH = 3-6 β-HCH = 0.04-0.12 δ-HCH = 0.02-0.08 γ-HCH = 1-21.3	0.43-0.87 (25 °C)	In water 8.52 (25 °C), 8.35 (pH 5, 25 °C) both in mgL^{-1}. In acetone >200, methanol 29-40, ethyl acetate <200, *n*-heptane 10-14 (all in gL^{-1}, 20 °C).	Insecticide with contact stomach and respiratory action. Mainly used for soil and seed treatment. Control of a broad spectrum of phyto-phagous and soil-inhabiting insects, public pests and animal ectoparasites. Used on a wide range of crops.	PHS listed under WFD as group 18 POP

Table A.1 (cont.) – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33].

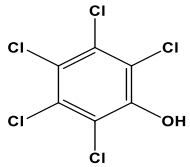
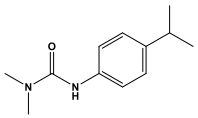
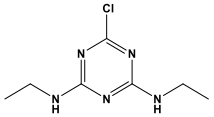
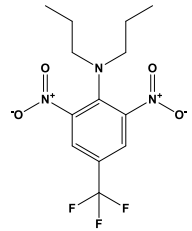
Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log K_{OW}	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification*
Pentachlorophenol C_6Cl_5OH CAS [87-86-5] Insecticide, Fungicide and Herbicide		266.3	210	4.0-5.1 (25 ºC)	15-23 (20 ºC)	0.0025-0.28 (25 ºC)	In water 14 mgL^{-1} (pH 4.5-5.5). Soluble in most organic solvents.	Insecticide, fungicide and non-selective contact herbicide. Used to control termites and frequently as an ester (pentachlorophenyl laureate) to protect wood from fungal rots and wood-boring insects and as a general herbicide. The sodium salt is used as a general disinfectant, e.g. for trays in mushroom houses.	PP listed under WFD as group 27
Isoproturon $C_{12}H_{18}N_2O$ CAS [34123-59-6] Urea Herbicide		206.3	1800-2400	2.87 (25 ºC)	3.3×10^{-3} (20 ºC)	1.24×10^{-5} (25 ºC)	In water 65 mgL^{-1} (22 ºC). In methanol 75, dichloro-methane 63, acetone 38, benzene 5 (all in gL^{-1}, 20 ºC).	Selective systemic herbicide, absorbed by roots and leaves. Photosynthetic electron transport inhibitor. Pre- and post-emergence control of annual grasses.	PP listed under WFD as group 19
Simazine $C_7H_{12}ClN_5$ CAS [122-34-9] 1,3,5-triazine Herbicide		201.7	5000	2.1	2.95×10^{-3} (25 ºC)	3.3×10^{-5} - 3.4×10^{-4} (25º C)	In water 6.2 mgL^{-1} (pH 7, 20 ºC). In ethanol 570, acetone 1500, toluene 130, *n*-octanol 390, *n*-hexane 3.1 (all in mgL^{-1}, 25 ºC). pKa 2.00.	Selective systemic herbicide absorbed principally through the roots, but also through the foliage. Used to control the most germinating annual grasses and broad-leaved weeds in pome fruit, stone fruit, bush and cane fruit, citrus fruit vines, strawberries nuts, olives, pineapples, field beans, French beans peas, maize, sweet corn, asparagus, hops, alfalfa, coffee, etc.	PP listed under WFD as group 29
Trifluralin $C_{13}H_{16}F_3N_3O_4$ CAS [1582-09-8] Dinitroaniline Herbicide		335.3	>10,000	4.83 (20 ºC)	$9.5\text{-}15\times10^{3}$ (20-25 ºC)	9.63 (20 ºC)	In water 0.184 (pH 5), 0.221 (pH 7), 0.189 (pH 9) (all in mgL^{-1}). In acetone, chloro-form, acetonitrile, toluene, ethyl acetate >1000, methanol 33-40, *n*-hexane 50-77 (all in gL^{-1}, 25 ºC).	Selective soil-herbicide. Pre-emergence control of many annual grasses and broad-leaved weeds in vines, strawberries, citrus fruit and in forestry. Used with linuron or isoproturon for control of annual grasses and broad-leaved weeds in winter cereals. Normally applied in pre-planting with soil incorporation. Post-planting is also possible for some crops.	PHS listed under WFD as group 33

Table A.1 (cont.) – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33].

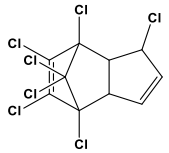
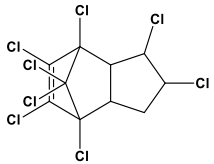
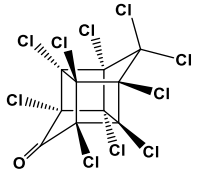
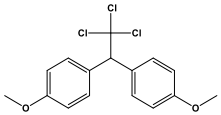
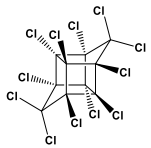
Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log Kow	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification*
Heptachlor $C_{10}H_5Cl_7$ CAS [76-44-8] Cyclodiene organochloride Insecticide		373.3	40	4.40-5.50	53 (20-25 °C)	112-845 (20-25 °C)	In water 0.056 mgL^{-1} (25-29 °C). Soluble in many organic solvents, e.g. in acetone 75, benzene 106, xylene 102, cyclohexanone 1190, carbon tetrachloride 1130, ethanol 450 (all in gmL^{-1}).	Non-systemic insecticide with contact, stomach and some respiratory action. Control of termites, ants and soil insects. Applied as a seed treatment, soil treatment or directly to foliage. Also used for control of household insects.	PP listed under WFD as group 44 POP
Chlordane $C_{10}H_6Cl_8$ CAS [57-74-9] Cyclodiene organochloride Insecticide		409.8	283	5.54	1.3 (25 °C)	2.92-9.5 (25 °C)	In water 0.1-1.83 mgL^{-1} (20-25 °C). Miscible with most aliphatic and aromatic organic solvents, including acetone.	Non-systemic insecticide with contact, stomach and respiratory action. Long residual activity. Used on land against insect pests. It also controls household insects, pests of man and domestic animals. Used in wood preservative, a protective treatment for underground cables and to reduce earthworm population in lawns. It may be applied to soil, directly to foliage or as seed treatment.	POP
Chlordecone $C_{10}Cl_{10}O$ CAS [143-50-0] Organochloride Insecticide		490.6	170	4.50	0.03 (25 °C)	3.11×10^{-3} (25 °C)	In water 2.70 mgL^{-1}. Slightly soluble in hydrocarbon solvents; soluble in alcohols, ketones, acetic acid.	Chlordecone was primarily used as an insecticide. Specific applications have included control of the banana root borer, application on non-fruit-bearing citrus trees to control rust mites, control of wireworms in tobacco fields, control of apple scab and powdery mildew, control of the grass mole cricket, and control of slugs, snails, and fire ants.	POP
Methoxychlor $C_{16}H_{15}Cl_3O_2$ CAS [72-43-5] Insecticide		345.7	5000	4.95-5.08	< 0.133 (20-25 °C)	1-1.6 (25 °C)	In water 0.1 mgL^{-1} (25 °C). Readily soluble in aromatic, chlorinated and ketonic solvents and vegetables oils. In chloroform and xylene 440, methanol 50, (all in gkg^{-1}, 22 °C)	Use as an insecticide for home and garden applications, livestock and poultry, alfalfa, soya beans, forests ornamental shrubs, deciduous fruits and nuts, and vegetables.	POP
Mirex $C_{10}Cl_{12}$ CAS [2385-85-5] Insecticide		545.5	235	5.28	0.11 (20-25 °C)	840-1013 (20 °C)	0.02 mgL^{-1} at 24 °C. In dioxane (15.3%), xylene (14.3%), benzene (12.2%), carbon tetrachloride (7.2%), methylethylketone (5.6%)	Used as fire retardant and pesticide.	POP

Table A.1 (cont.) – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33].

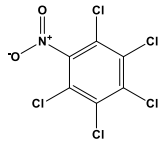
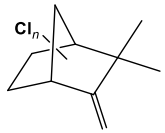
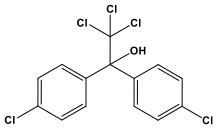
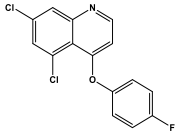
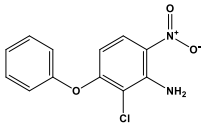
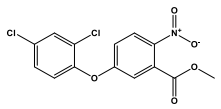
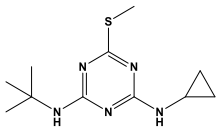
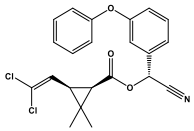
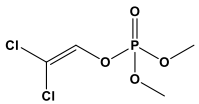
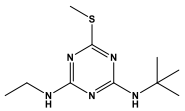
Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log Kow	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification*
Quintozene $C_6Cl_5NO_2$ CAS [82-68-8] Chlorophenyl Fungicide		295.3	>5000	5.1	12.7 (25 °C)	0.37-0.48 (25 °C)	In water 0.1 mgL^{-1} (20 °C). In toluene 1140, methanol 20, heptanes 30 (all in gL^{-1})	Seed and soil contact fungicide. Control of damping-off diseases in brassicas, lettuce, cotton, flower crops, tomatoes, etc. Also used on peanuts, bananas, beans, peas, rice maize, safflowers, sorghum, soya beans, etc.	POP
Toxaphene $C_{10}H_{10}Cl_8$ (approximately) CAS [8001-35-2] Organochloride Insecticide		414 (average)	40	3.2-5.5	0.13-0.53 (20-25 °C)	0.42-6382 (20-25 °C)	In water 0.3-3 mgL^{-1}	Toxaphene is a non-systemic and contact insecticide that was used primarily on cotton, cereal grains fruits, nuts and vegetables. It has also been used to control ticks and mites in livestock. Toxaphene is a mixture of at least 670 chlorinated bicyclic terpenes.	POP
Dicofol $C_{14}H_9Cl_5O$ CAS [115-32-2] Organochloride Acaricide		370.5	575	3.5-4.3	5.3×10^{-3} (25 °C)	5.7×10^{-5} (25 °C)	In water 0.8 mgL^{-1} (25 °C). In toluene 400, methanol 36, isopropanol 30 (all in gL^{-1}, 25 °C).	Non-systemic acaricide with contact action and with little insecticidal activity. Recommended for control of many species of phytophagous mite on a wide range of crops (fruit, vines, ornamentals, vegetables and field crops).	PHS listed under WFD as group 34
Quinoxyfen $C_{15}H_8Cl_2FNO$ CAS [124495-18-7] Phenoxyquinoline Fungicide		308.1	>5000	4.60 (20 °C)	2.0×10^{-2} (25 °C)	3.19×10^{-2} (25 °C)	In water 0.047 mgL^{-1} at pH 7 and 0.128 mgL^{-1} at pH 5. In methanol 21.5, acetone 116 (all in gL^{-1}, 25 °C).	Use to control cereal and grape powdery mildew. Offers long-term protection (up to 70 days).	PHS listed under WFD as group 36
Aclonifen $C_{12}H_9ClN_2O_3$ CAS [74070-46-5] Diphenyl ether Herbicide		264.7	5596	4.37	1.6×10^{-2} (20 °C)	3.2×10^{-3} (20 °C)	In water 1.4 mgL^{-1} (20 °C). In methanol 50, *n*-hexane 4.5, toluene 390 (all in gkg^{-1}, 20 °C).	Systemic selective herbicide. Pre-emergence control of grass and broad-leaved weeds in winter wheat, potatoes, sunflowers, peas, carrots, maize and other crops.	PP listed under WFD as group 38

Table A.1 (cont.) – Pesticides classified as priority substances under Annex X of the WFD and some of the most relevant physicochemical properties [1, 7, 12, 22, 33].

Common name	Chemical structure	MW	LD_{50} ($mgkg^{-1}$)	Log k_{OW}	VP (mPa)	H ($Pa\ m^3mol^{-1}$)	Solubility	Mode of action and uses	Classification*
Bifenox $C_{14}H_9Cl_2NO_5$ CAS [42576-02-3] Diphenyl ether Herbicide		342.1	>6400	4.5-5.6	0.32 (20-25 °C)	0.011-0.32 (20-25 °C)	In water 0.35 mgL^{-1} (25 °C). In acetone 400, chlorobenzene 400, xylene 300, ethanol <50 (all in gkg^{-1}, 25 °C). Slight soluble in aliphatic hydrocarbons.	Selective herbicide absorbed by the foliage emerging shoots and roots. Control of annual broad-leaved weeds and some grasses in cereals, maize, some other crops. Applied pre-plant incorporated, pre-herbicides to extend the spectrum of activity.	PP listed under WFD as group 39
Cybutryne $C_{11}H_{19}Cl_3N_5S$ CAS [28159-98-0] Triazine Algicide also known as Irgarol		253.37	5596	3.1-3.2	0.03 (25 °C)	4.1×10^{-4}	In water 9.0 mgL^{-1} (pH 7, 20 °C). In *n*-hexane 2.0 gL^{-1} (20 °C) and methanol 50.58 gL^{-1}(20 °C)	Cybutryne is used as antifouling paints with increasing popularity since restriction of the use of tributyltin. Cybutryne is known to inhibit photosynthesis. This is in line with the observation that primary producers (algae and aquatic macrophytes) were the most sensitive aquatic species. The active substance, however, appeared to be also highly toxic to fish and invertebrates.	PP listed under WFD as group 40
Cypermethrin $C_{22}H_{19}Cl_2N_2O_3$ CAS [52315-07-8] Pyrethroid Insecticide		416.3	247	5.3-5.6	2.3×10^{-4} (20 °C)	0.08 (25 °C)	In water 4 μgL^{-1} (pH 7). In acetone 450, ethyl acetate 2000, ethanol 337, *n*-hexane 142 (all in gL^{-1}, 20 °C).	Non-systemic insecticide with contact and stomach action. Good residual activity on treated plants. Used to control a wide range of insects in fruit, vines, vegetables, potatoes, lettuce, tomatoes, etc. Control of flies and other insects in animal houses and mosquitoes and other insects pests in public health.	PP listed under WFD as group 41
Dichlorvos $C_4H_7Cl_2O_4P$ CAS [62-73-7] Organophosphorus Insecticide, acaricide		221.0	25	1.4-2.3	2.1×10^{3} (25 °C)	0.097-506 (20-25 °C)	In water 10 gL^{-1} (25 °C). Completely miscible with aromatic hydrocarbons, chlorinated hydrocarbons and alcohols.	Cholinesterase inhibitor. Insecticide and acaricide with respiratory, contact and stomach action. Gives rapid knockdown. Use to control of household and public health insects' pests. Used as insecticide in crops (fruit, vines, vegetables, etc.).	PP listed under WFD as group 42
Terbutryn $C_{10}H_{19}N_5S$ CAS [886-50-0] 1,3,5-triazine Herbicide		241.4	2100	3.4-3.7	0.28 (20-25 °C)	1.3×10^{-3} (20 °C)	In water 22 mgL^{-1} (20-25 °C). In acetone 220, *n*-hexane 9, *n*-octanol 130, methanol 220, toluene 45 (all in gL^{-1}, 20 °C). pKa 4.07-4.30.	Selective herbicide absorbed by the roots. Used pre-emergence in winter cereals to control blackgrass and annual meadow grass. In mixture with terbuthylazine used on beans, peas and potatoes.	PP listed under WFD as group 45

* - Not define as a priority substance but one of the other pollutants legislated by other earlier Directives and further included in the list of PS under Annex X WFD. ** - CAS – Chemical Abstract System number; LD_{50} – acute oral LD_{50} for rats. The LD_{50} (lethal dosage) value of a pesticide, or any other toxic substance, is a statistical estimate of the dosage necessary to kill 50 percent of a population of test animals (usually white rats) with a single exposure under standardized conditions in the laboratory. It is expressed in milligrams of poison per kilogram of body weight ($mgkg^{-1}$) for rodents or micrograms per gram (mgg^{-1}) for insects. $Log k_{OW}$ – logarithm of octanol-water partition coefficient; VP – vapor pressure; H – Henry's Laws constant; POP – Persistent organic pollutant; PS – Priority substance; PHS – Priority hazardous substance.

B

Supplementary Information -Annex B

Chapter 3 and Chapter 4

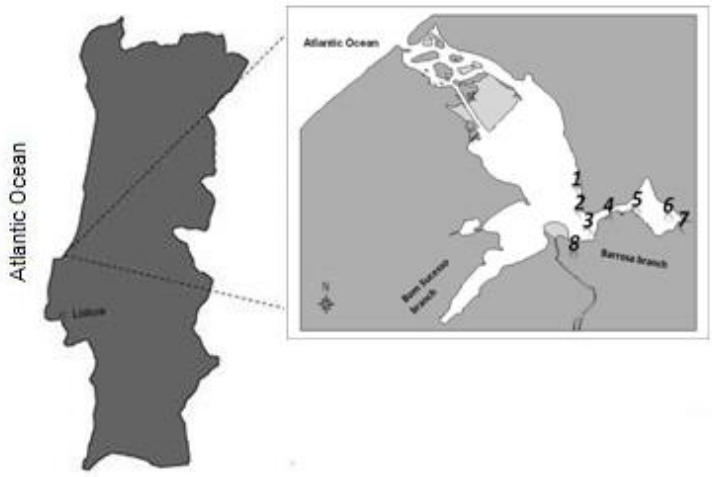


Fig. B.1 – Location of sampling sites in Óbidos Lagoon.

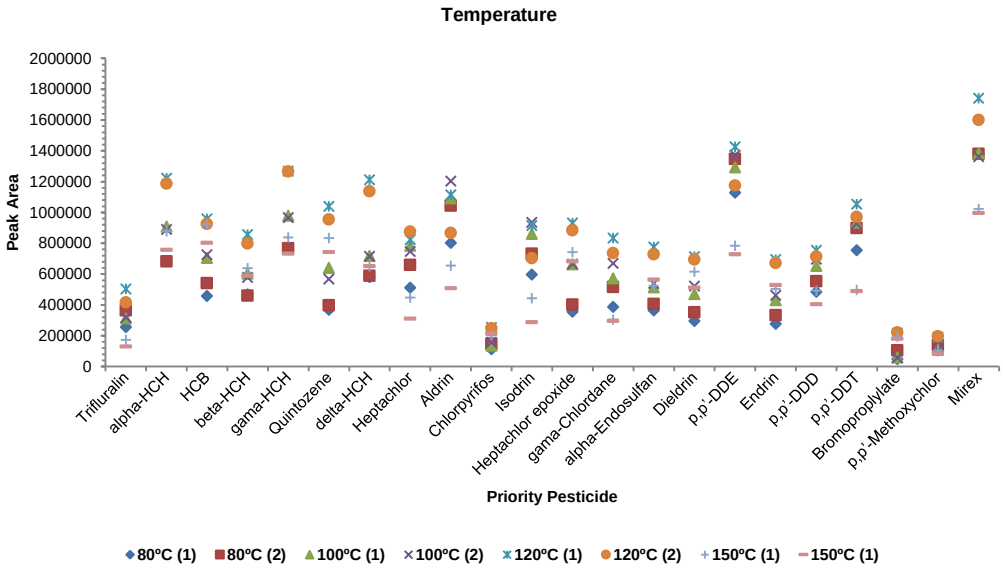


Fig. B.2 – Influence of the temperature on the extraction of the all the studied PPs. Two replications were carried out and both values were presented for a better overview of the differences between the two replications. The values of the replications were represented by (**1**) for the first replication and (**2**) for the second replication.

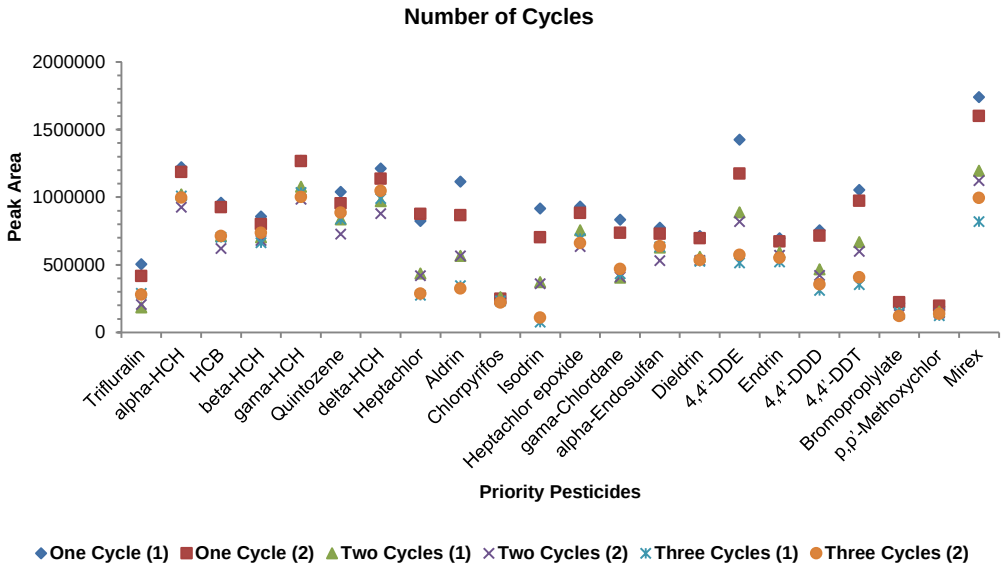


Fig. B.3 – Influence of the number of cycles on the extraction of the all the studied PPs. Two replications were carried out and both values were presented for a better overview of the differences between the two replications. The values of the replications were represented by (**1**) for the first replication and (**2**) for the second replication.

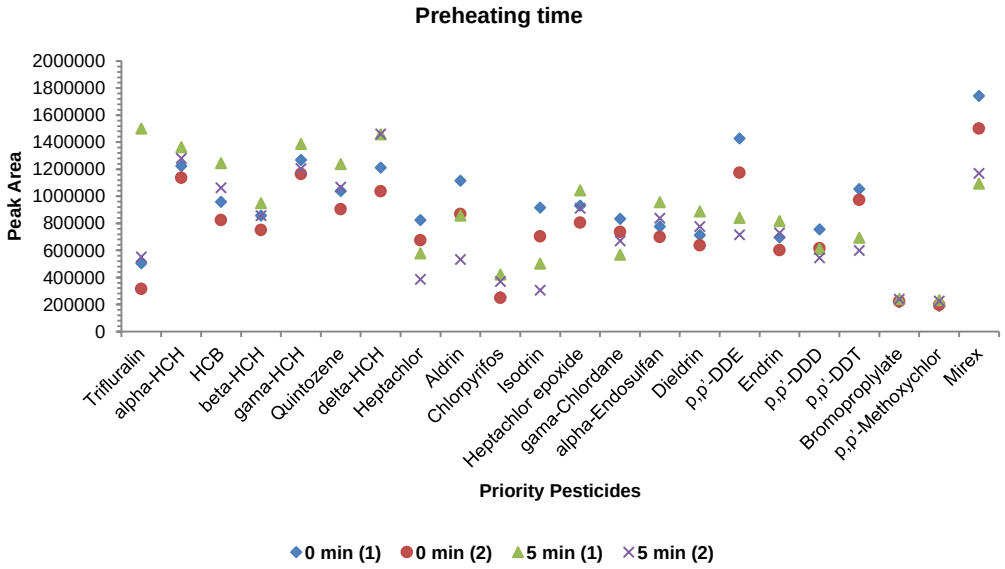


Fig. B.4 – Influence of the preheating time on the extraction of the all the studied PPs. Two replications were carried out and both values were presented for a better overview of the differences between the two replications. The values of the replications were represented by (**1**) for the first replication and (**2**) for the second replication

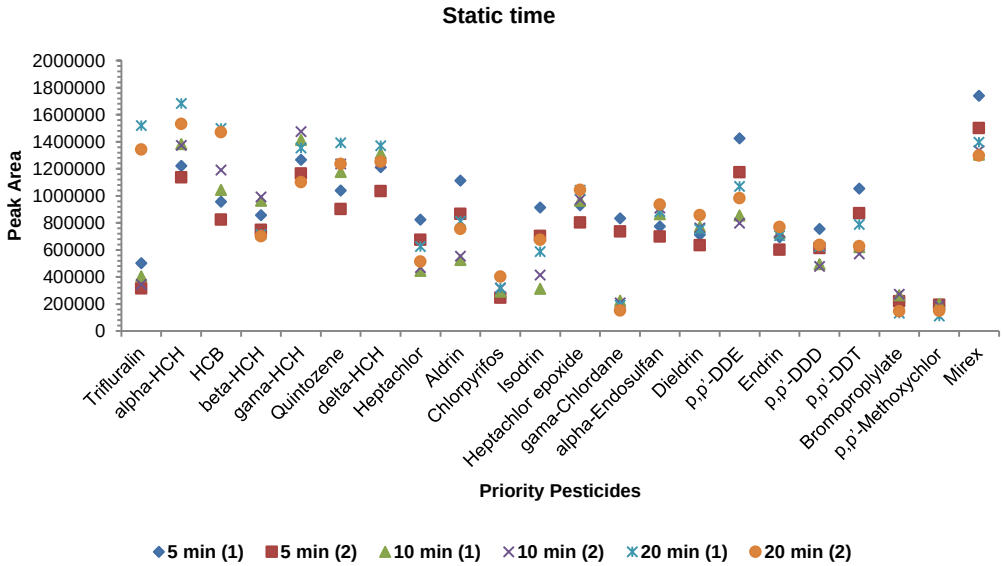


Fig. B.5 – Influence of the static time on the extraction of the all the studied PPs. Two replications were carried out and both values were presented for a better overview of the differences between the two replications. The values of the replications were represented by (**1**) for the first replication and (**2**) for the second replication

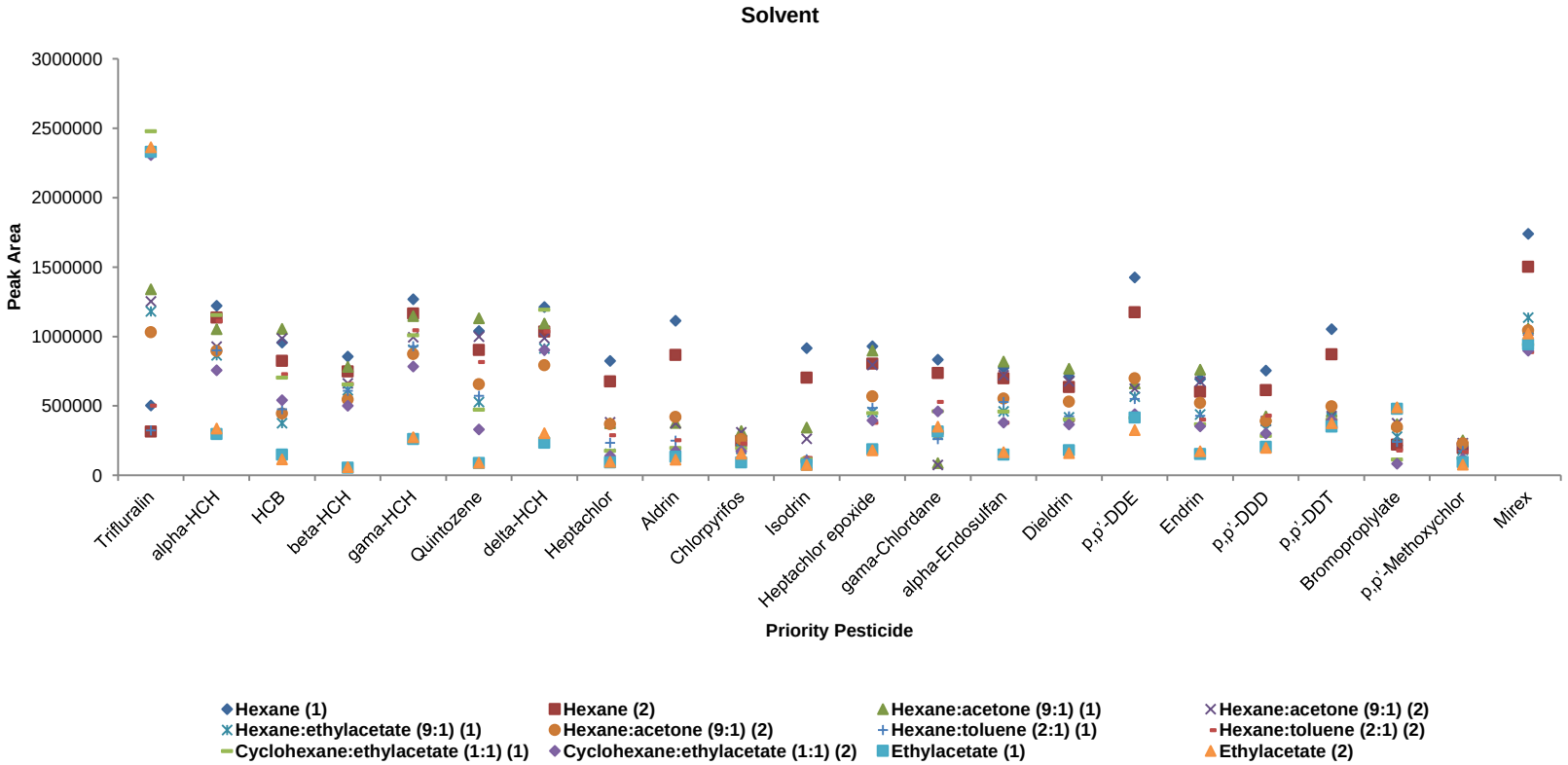


Fig. B.6 – Influence of solvent on the extraction of the all the studied PP. Two replications were carried out and both values were presented for a better overview of the differences between the two replications. The values of the replications were represented by (**1**) for the first replication and (**2**) for the second replication.

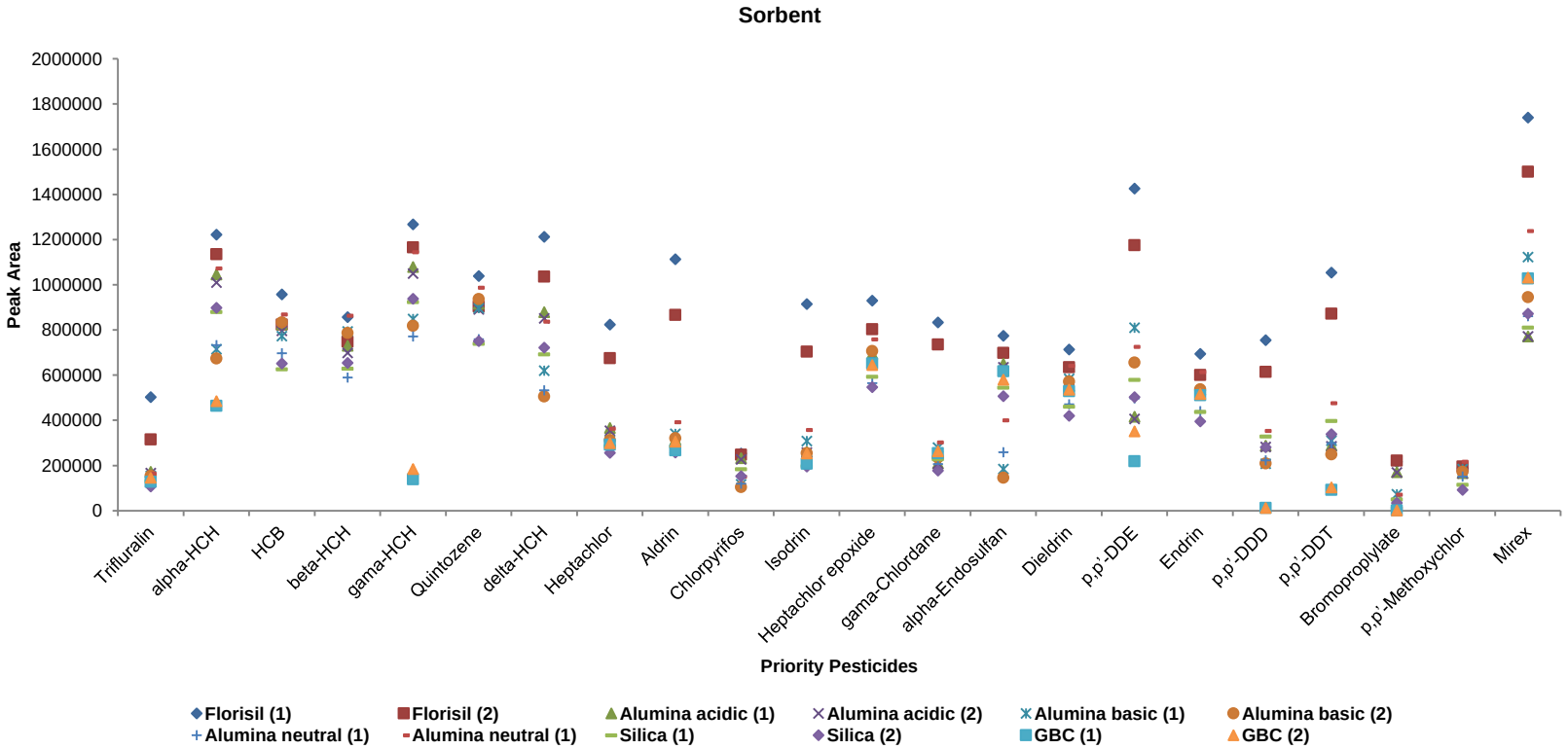


Fig. B.7 – Effect of in-cell reagents on the selective of pesticides extraction: Influence of sorbent on sample clean-up. Two replications were carried out and both values were presented for a better overview of the differences between the two replications: (**1**) is the peak area of the first replication and (**2**) the peak area of the second replication.

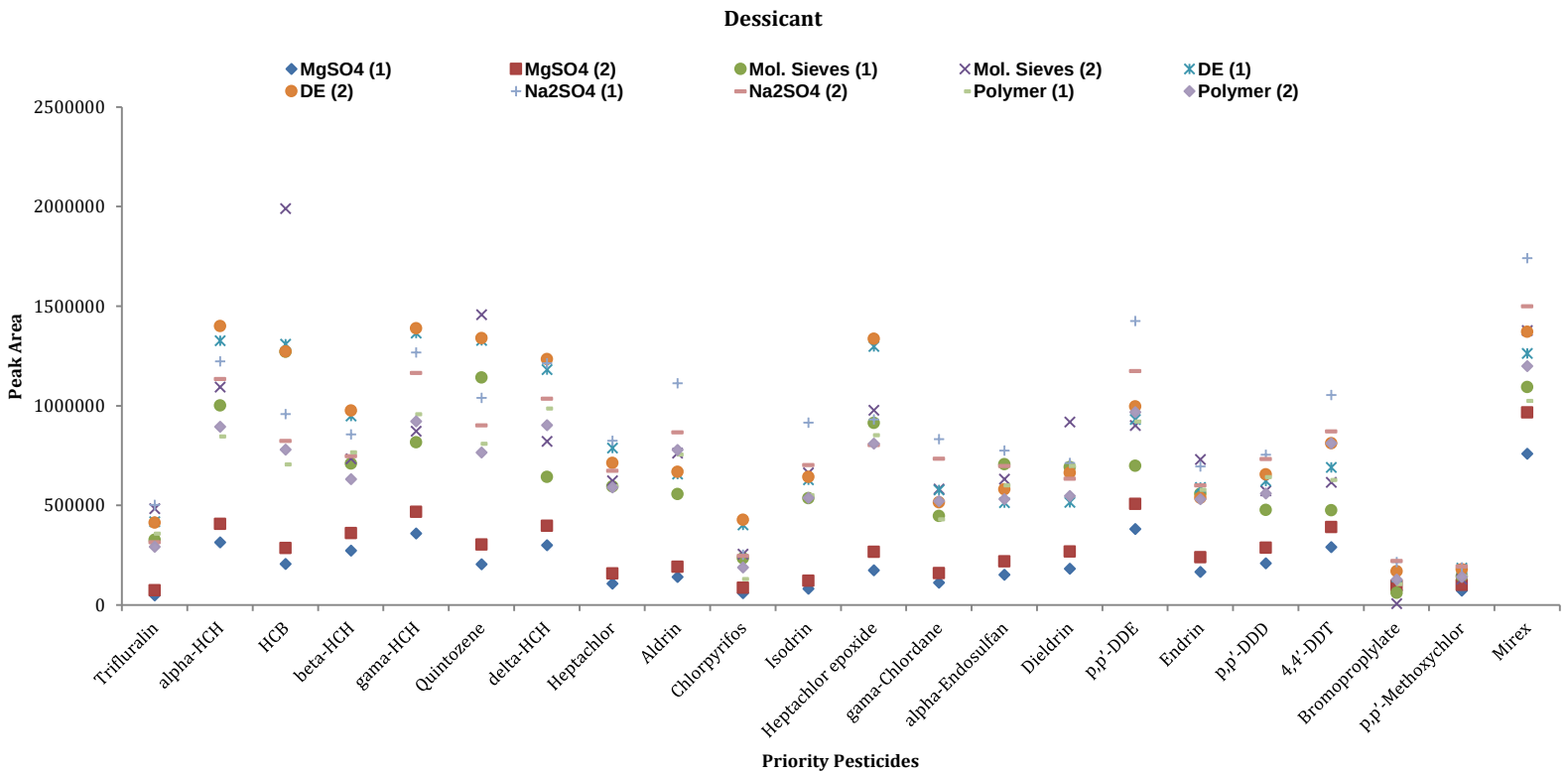


Fig. B.8 – Effect of in-cell reagents on the selective of pesticides extraction: Influence of the desiccant on sample drying. Two replications were carried out and both values were presented for a better overview of the differences between the two replications: (**1**) is the peak area of the first replication and (**2**) the peak area of the second replication. Mol. Sieves (molecular sieves); DE (diatomaceous earth).

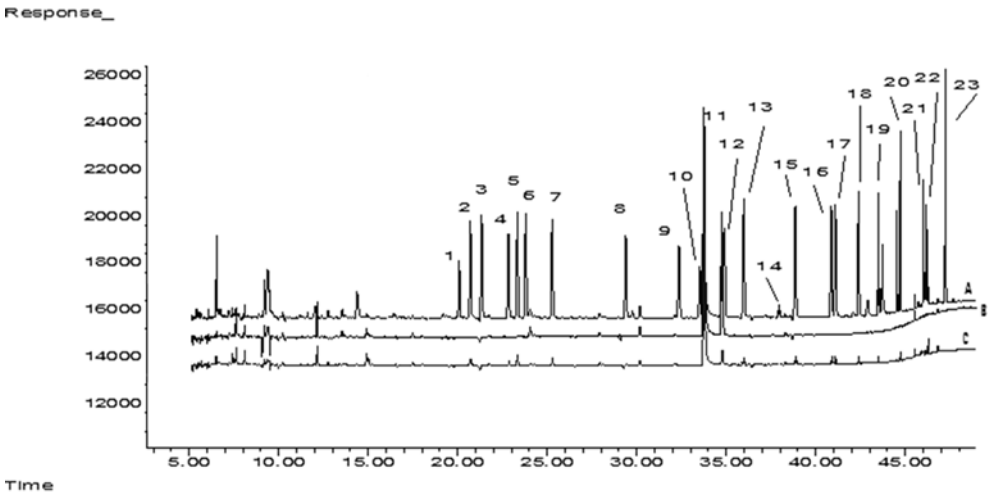


Fig. B.9 – Chromatograms of *Ulva* sp. samples collected in the Bom Sucesso branch of Óbidos Lagoon: **A** – spiked (25 ng g^{-1}) sample; **B** – unspiked sample collected during the Summer time; **C** – unspiked sample collected during the Winter time. Peak identification: 1 – trifluralin (20.28 min), 2 – α-HCH (20.72 min), 3 - HCB (21.34 min), 4 - β-HCH (22.83 min), 5 - γ-HCH (23.34 min), 6 – quintozene (23.80 min), 7 - δ-HCH (25.28 min), 8 – heptachlor (29.39 min), 9 – aldrin (32.37 min), 10 – chlorpyrifos (33.53 min), 11 – *p,p'*-dibromobiphenyl (Internal Standard, 33.76 min), 12 – isodrin (34.92 min), 13 – heptachlor epoxide (35.99 min), 14 – γ-chlordane (37.98 min), 15 – α-endosulfan (38.87 min), 16 – dieldrin (40.88 min), 17 – *p,p´*-DDE (41.41 min), 18 – endrin (42.40 min), 19 – *p,p´*-DDD (43.50 min), 20 – *p,p´*-DDT (44.72 min), 21 – bromopropylate (46.01 min), 22 – *p,p´*-methoxychlor (46.18 min), 23 – mirex (47.24 min).

Supplementary Information - Annex C

Chapter 6

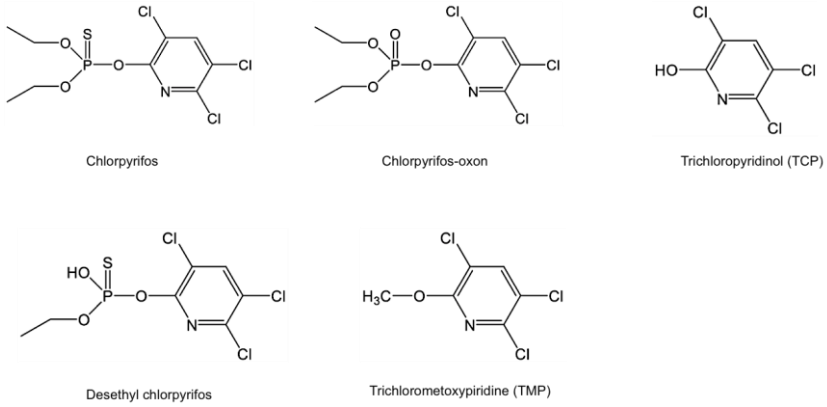


Fig. C.1 – Chemical structure of chlorpyrifos and its degradation products.

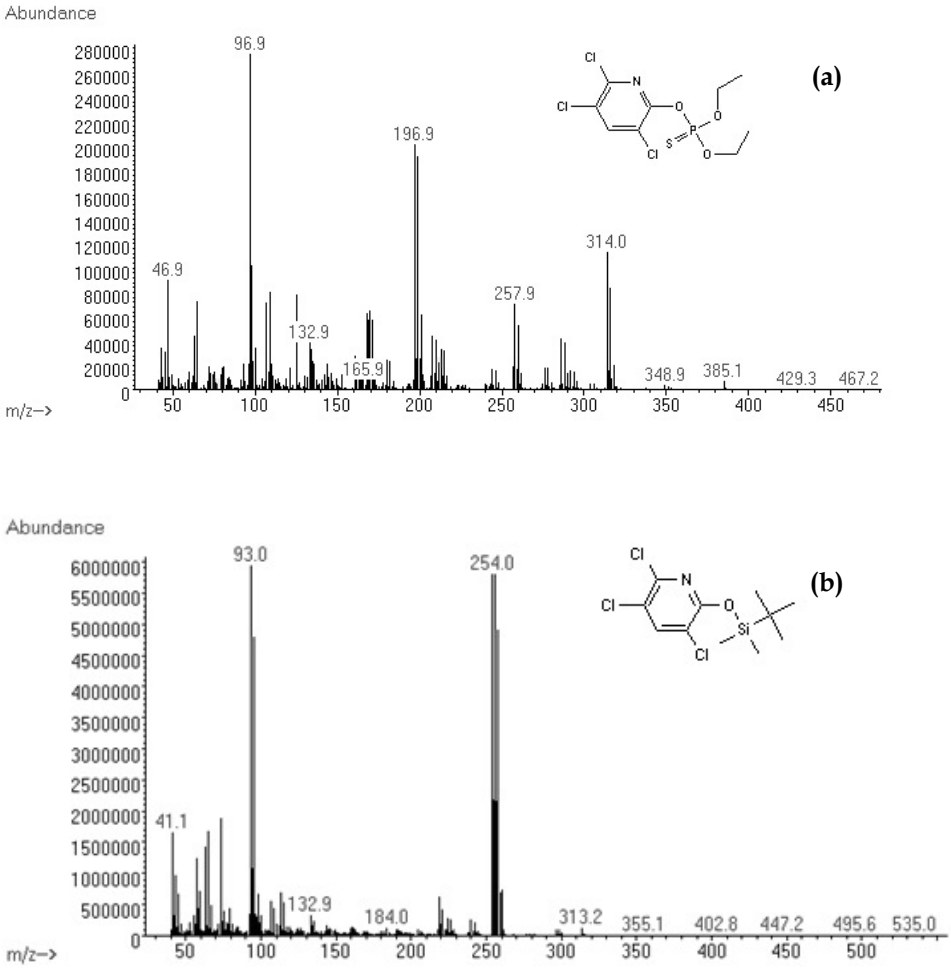


Fig. C.2 – (**a**) Mass spectrum of chlorpyrifos (*m/z* 349: $[M]^+$; *m/z* 314: $[M\text{-}Cl]^+$; *m/z* 258: [M-Cl-2 alkyl ester bonds]$^+$; *m/z* 197: [trichloropyriol]$^+$); (**b**) mass spectrum of 3,5,6-trichloro-2-pyridinol as the *tert*-butyl-dimethylsilyl derivative (*m/z* 311: $[M]^+$; *m/z* 254: [M-butyl]$^+$; *m/z* 93: [M- *tert*-butyl-dimethylsilyl-3Cl]$^+$).

Supplementary Information - Annex D

Chapter 7

Fig. D.1 – Sampling in the Western coast of Portugal: Óbidos Lagoon, site **1** – Foz do Arelho (39º25'41.25''N 9º13'22.10''W); site **2** – Barrosa branch (39º24'13.56''N 9º11'51.60''W) and site **3** – Bom Sucesso branch (39º23'13.53''N 9º13'35.72''W) (image source: Google Earth).

Fig. D.2 – Sampling in Irvine, CA: Newport Back Bay (NPBB), site **A** (33º37'3.36''N 117º54'13.44''W); site **B** (33º36'29.58''N 117º54'16.02''W); site **C** (33º37'6.06''N 117º55'32.28''W) and San Joaquin Wetlands at the inlet (33º39'35.60'N 117º50'31.20''W) and outflow (33º39'50.10''N 117º50'51.00''W) (image source: Google Earth).

Table D.1 – pH and salinity of water samples collected in Óbidos Lagoon (OL), Newport Back Bay (NPBB) and San Joaquim Irvine Ranch Water District (SJ IRWD).

Water	pH	Salinity
OL Site 1	7.6	35.6
OL Site 2	7.8	34.0
OL Site 3	7.4	25.4
NPBB A	7.7	36.1
NPBB B	7.5	35.7
NPBB C	7.8	35.9
SJ IRWD inlet	8.3	-
SJ IRWD outflow	8.2	-

Table D.2 – HPLC Gradient 1 used in the analysis of the MeOH:H_2O (1:1) PPL I fraction. Flow rate was 1 $mLmin^{-1}$.

Time (min)	Acetonitrile (%)	MilliQ® water with 0.2% acetic acid (%)
0.00	2.0	98.0
20.00	12.5	87.5
25.00	12.5	87.5
28.00	25.0	75.0
33.00	25.0	75.0
38.00	50.0	50.0
48.00	50.0	50.0
50.00	100.0	0.0
55.00	100.0	0.0
60.00	2.0	98.0
70.00	2.0	98.0

Table D.3 – HPLC Gradient 2 used in the analysis of the PPL II MeOH:ACN:H_2O (1:1:2) and the PPL III MeOH:ACN (1:1) fractions. Flow rate was 1 $mLmin^{-1}$.

Time (min)	Acetonitrile (%)	MilliQ® water with 0.2% acetic acid (%)
0.00	2.0	98.0
1.00	2.0	98.0
5.00	12.5	87.5
10.00	12.5	87.5
13.00	25.0	75.0
18.00	25.0	75.0
23.00	50.0	50.0
33.00	50.0	50.0
35.00	100.0	0.0
40.00	100.0	0.0
45.00	2.0	98.0
50.00	2.0	98.0

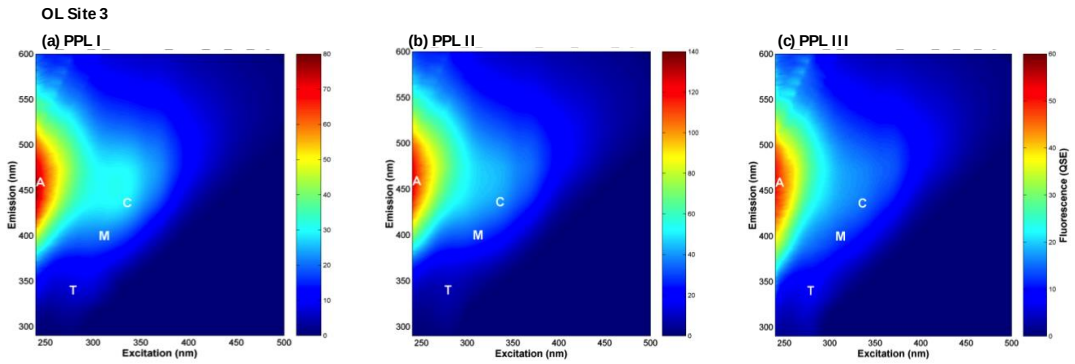


Fig. D.3 – EEMs of the PPL fractions of Óbidos Lagoon site 3: (a) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

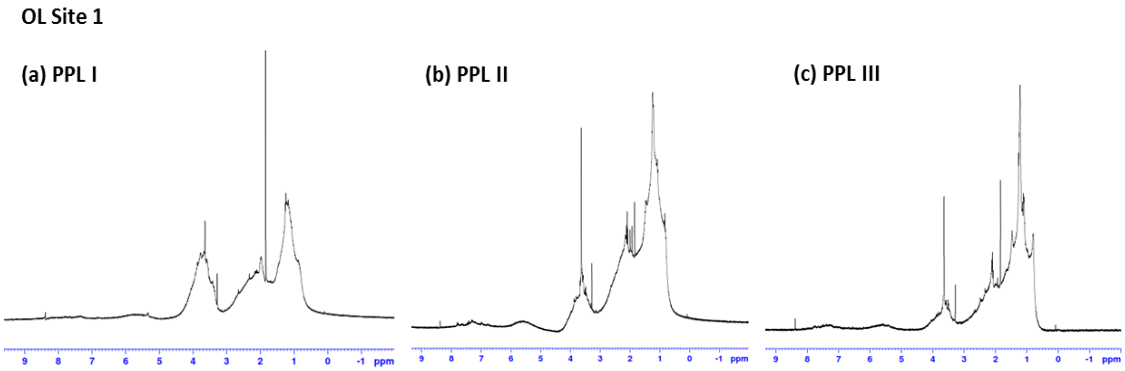


Fig. D.4 – ^{1}H NMR spectra of the PPL fractions of Óbidos Lagoon site 1: (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

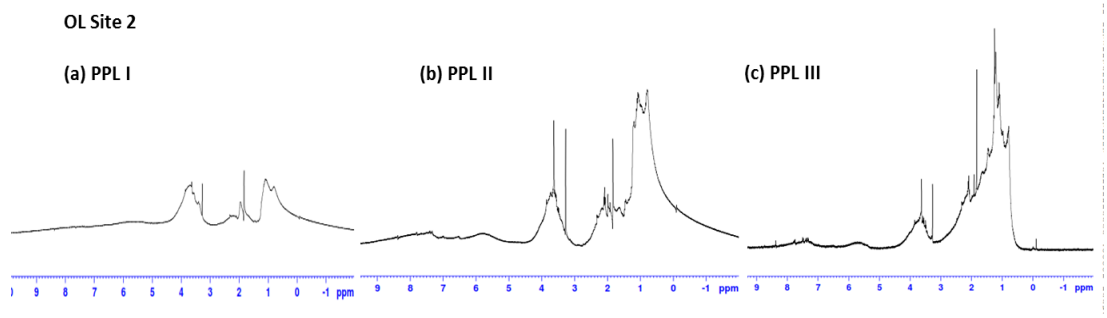


Fig. D.5 – ^{1}H NMR spectra of the PPL fractions of Óbidos Lagoon site 2: (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

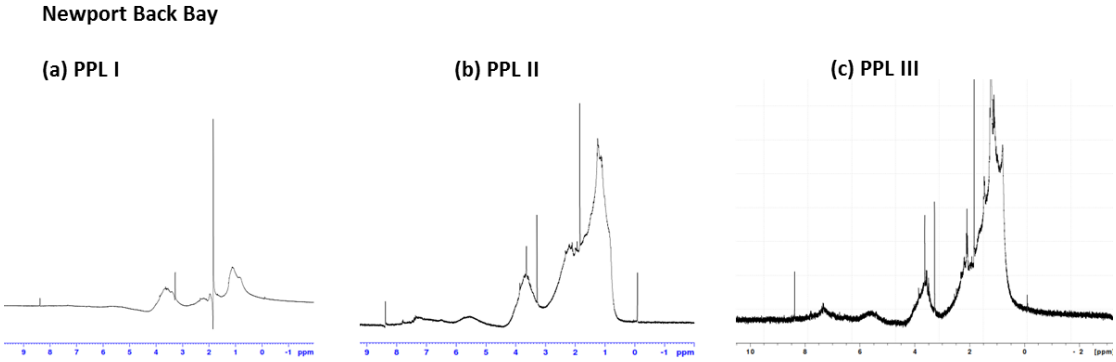


Fig. D.6 – ^{1}H NMR spectra of the PPL fractions of Newport Back Bay: (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

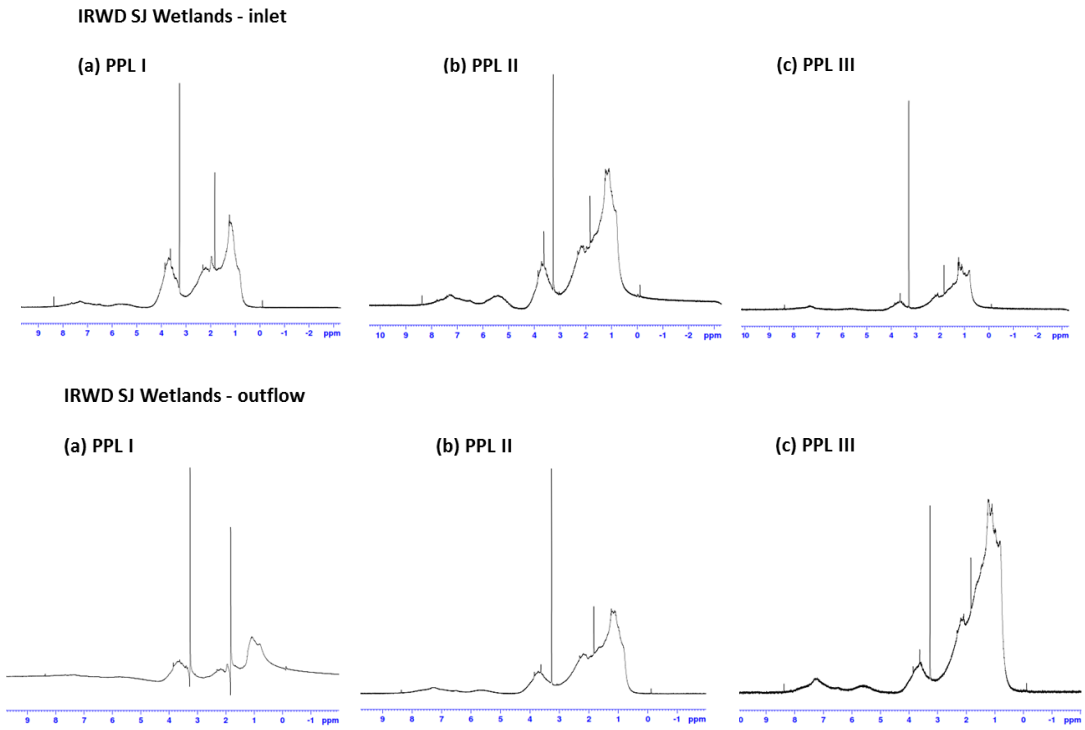


Fig. D.7 – ^{1}H NMR spectra of the PPL fractions of San Joaquin IRWD wetlands inlet and outflow: (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

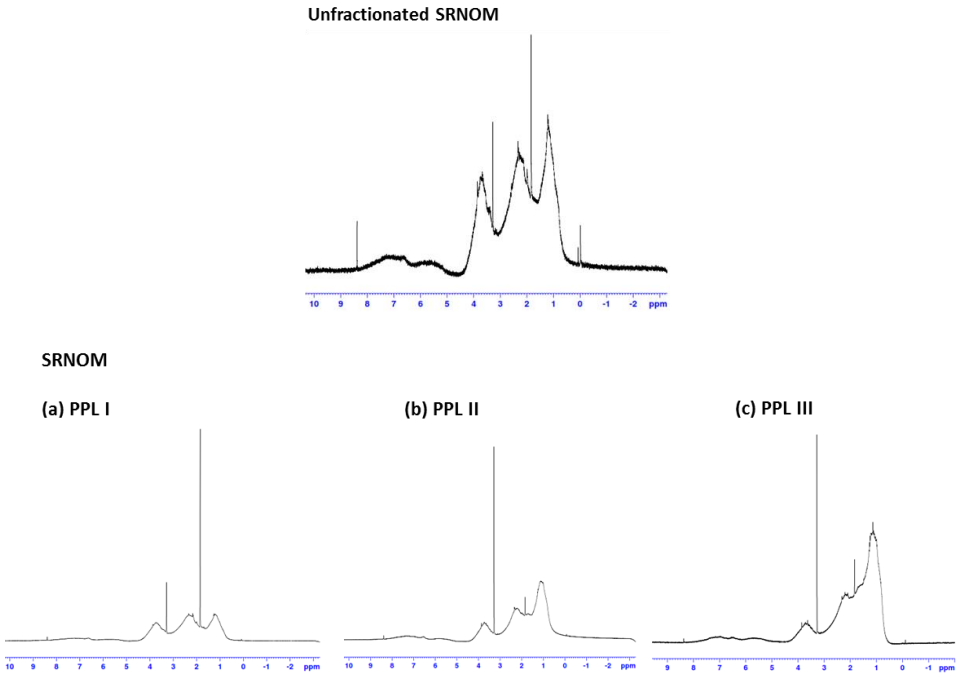


Fig. D.8 – ^{1}H NMR spectra of unfractionated SRNOM and fractionated SRNOM (PPL fractions): (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

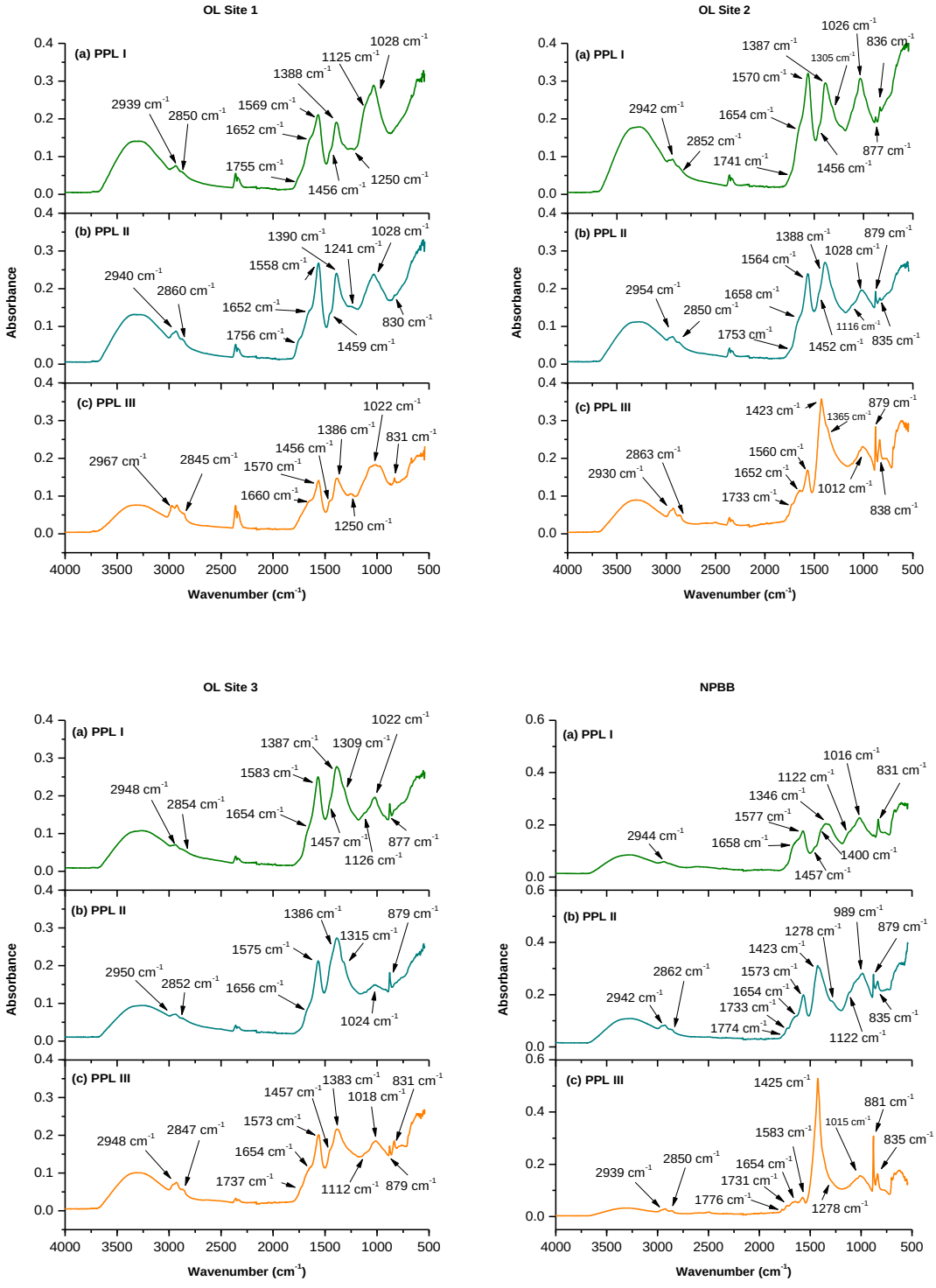


Fig. D.9 – FTIR spectra of PPL fractions of OL Site 1, OL Site 2, OL Site 3 and NPBBB: (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).

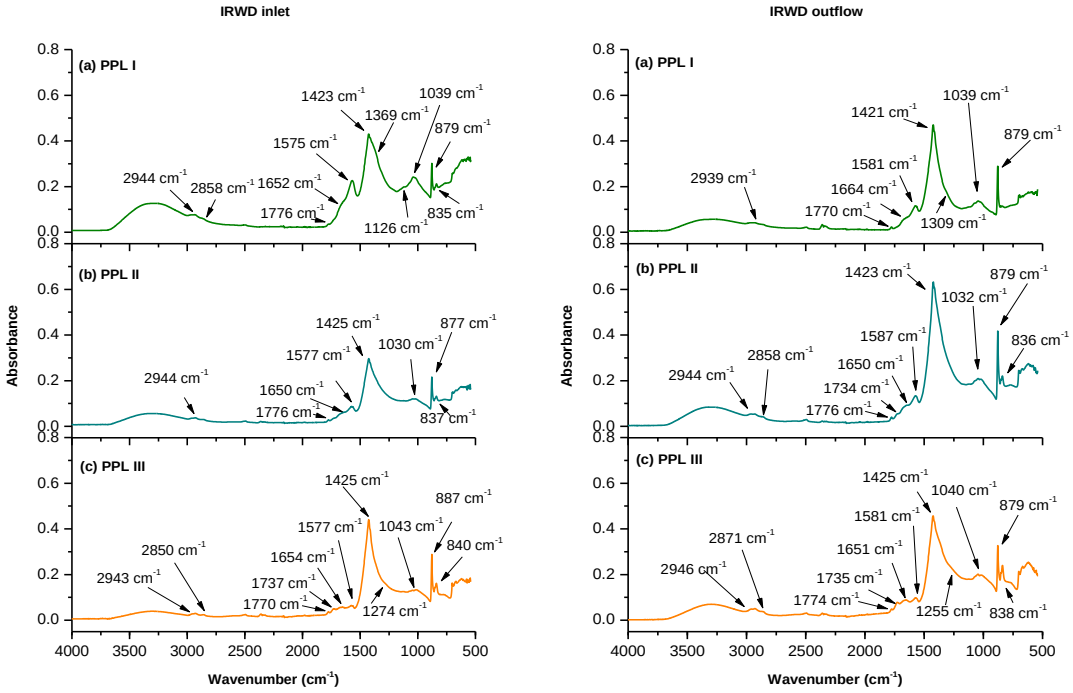


Fig. D.10 – FTIR spectra of PPL fractions of IRWD inlet and IRWD outflow: (**a**) PPL I = MeOH:H_2O (1:1), (**b**) PPL II = MeOH:ACN:H_2O (1:1:2); (**c**) PPL III = MeOH:ACN (1:1).